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(54) Title: FLAVIVIRUS RECOMBINANT POXVIRUS VACCINE															

(57) Abstract

What is described is a recombinant poxvirus, such as vaccinia virus, smallpox virus and canarypox virus, containing foreign DNA from flavivirus, such as Japanese encephalitis virus, yellow fever virus and Dengue virus. In a preferred embodiment, the recombinant poxvirus generates an extracellular particle containing flavivirus E and M proteins capable of inducing neutralizing antibodies, hemagglutination-inhibiting antibodies and protective immunity against flavivirus infection. What is also described is a vaccine containing the recombinant poxvirus for inducing an immunological response in a host animal inoculated with the vaccine.

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FLAVIVIRUS RECOMBINANT POXVIRUS VACCINE

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 07/714,687, filed June 13, 1991, which is a continuation-in-part of application Serial No. 5 07/711,429, filed June 6, 1991, which in turn is a continuation of application Serial No. 07/567,960, filed August 15, 1990.

FIELD OF THE INVENTION

The present invention relates to a modified 10 poxvirus and to methods of making and using the same. More in particular, the invention relates to recombinant poxvirus, which virus expresses gene products of a flavivirus gene, and to vaccines which provide protective immunity against flavivirus infections.

15 Several publications are referenced in this application. Full citation to these references is found at the end of the specification preceding the claims. These references describe the state-of-the-art to which this invention pertains.

20 BACKGROUND OF THE INVENTION

Vaccinia virus and more recently other poxviruses have been used for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into live infectious poxvirus involves recombination between pox 25 DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus (Piccini et al., 1987).

Specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to 30 the methods for creating synthetic recombinants of the vaccinia virus described in U.S. Patent No. 4,603,112, the disclosure of which patent is incorporated herein by reference.

First, the DNA gene sequence to be inserted into 35 the virus, particularly an open reading frame from a non-pox source, is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has

been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1986).

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, in a nonessential region of its genome, of foreign DNA sequences. The term "foreign" DNA designates exogenous DNA, particularly DNA from a non-pox source, that codes for gene products not ordinarily produced by the genome into which the exogenous DNA is placed.

Genetic recombination is in general the exchange of homologous sections of DNA between two strands of DNA. In certain viruses RNA may replace DNA. Homologous sections of nucleic acid are sections of nucleic acid (DNA or RNA) which have the same sequence of nucleotide bases.

Genetic recombination may take place naturally during the replication or manufacture of new viral genomes within the infected host cell. Thus, genetic recombination between viral genes may occur during the viral replication cycle that takes place in a host cell which is co-infected with two or more different viruses or other genetic constructs. A section of DNA from a first genome is used interchangeably in constructing the section of the genome of a second co-infecting virus in which the DNA is homologous with that of the first viral genome.

However, recombination can also take place between sections of DNA in different genomes that are not perfectly homologous. If one such section is from a first genome

homologous with a section of another genome except for the presence within the first section of, for example, a genetic marker or a gene coding for an antigenic determinant inserted into a portion of the homologous DNA, recombination can still take place and the products of that recombination are then detectable by the presence of that genetic marker or gene in the recombinant viral genome.

Successful expression of the inserted DNA genetic sequence by the modified infectious virus requires two conditions. First, the insertion must be into a nonessential region of the virus in order that the modified virus remain viable. The second condition for expression of inserted DNA is the presence of a promoter in the proper relationship to the inserted DNA. The promoter must be placed so that it is located upstream from the DNA sequence to be expressed.

The family Flaviviridae comprises approximately 60 arthropod-borne viruses that cause significant public health problems in both temperate and tropical regions of the world (Shope, 1980; Monath, 1986). Although some highly successful inactivated vaccines and live-attenuated vaccines have been developed against some of these agents, there has been a recent surge in the study of the molecular biology of flaviviruses in order to produce recombinant vaccines to the remaining viruses, most notably dengue (Brandt, 1988).

Flavivirus proteins are encoded by a single long translational open reading frame (ORF) present in the positive-strand genomic RNA. The genes encoding the structural proteins are found at the 5' end of the genome followed by the nonstructural glycoprotein NS1 and the remaining nonstructural proteins (Rice et al., 1985). The flavivirus virion contains an envelope glycoprotein, E, a membrane protein, M, and a capsid protein, C. In the case of Japanese encephalitis virus (JEV), virion preparations usually contain a small amount of the glycoprotein precursor to the membrane protein, prM (Mason et al., 1987a). Within JEV-infected cells, on the other hand, the M protein is

present almost exclusively as the higher molecular weight prM protein (Mason et al., 1987a; Shapiro et al., 1972).

Studies that have examined the protective effect of passively administered monoclonal antibodies (MAbs) 5 specific for each of the three flavivirus glycoproteins (prM, E, NS1) have demonstrated that immunity to each of these antigens results in partial or complete protection from lethal viral challenge. Monoclonal antibodies to E can provide protection from infection by Japanese encephalitis 10 virus (JEV) (Kimura-Kuroda et al., 1988; Mason et al., 1989), dengue type 2 virus (Kaufman et al., 1987) and yellow fever virus (YF) (Gould et al., 1986). In most cases, passive protection has been correlated with the ability of 15 these E MAbs to neutralize the virus *in vitro*. Recently, Kaufman et al. (1989) have demonstrated that passive protection can also be produced with prM MAbs that exhibit weak or undetectable neutralizing activity *in vitro*. The 20 ability of structural protein specific MAbs to protect animals from infection is consistent with the conventional hypothesis that structural protein antibodies attenuate viral infection by blocking virus binding to target cells. Passive protection experiments using MAbs to the NS1 protein 25 of yellow fever virus (Schlesinger et al., 1985; Gould et al., 1986) and dengue type 2 virus (Henchal et al., 1988) have demonstrated that antibodies to this nonstructural glycoprotein can protect animals from lethal viral 30 infection. Since these MAbs do not exhibit viral binding properties, their protection is presumably mediated by some less conventional mechanism of attenuation of viral infection (Gibson et al., 1988).

Additional support for the ability of NS1 immunity to protect the host from infection comes from direct immunization experiments in which NS1 purified from either 35 yellow fever virus-infected cells (Schlesinger et al., 1985, 1986) or dengue type 2 virus-infected cells (Schlesinger et al., 1987) induced protective immunity from infection with the homologous virus.

Although significant progress has been made in deriving the primary structure of these three flavivirus glycoprotein antigens, less is known about their three-dimensional structure. The ability to produce properly 5 folded, and possibly correctly assembled, forms of these antigens may be important for the production of effective recombinant vaccines. In the case of NS1-based vaccines, dimerization of NS1 (Winkler et al., 1988) may be required to elicit the maximum protective response. For the E 10 protein, correct folding is probably required for eliciting a protective immune response since E protein antigens produced in *E. coli* (Mason et al., 1989) and the authentic E protein prepared under denaturing conditions (Wengler et al., 1989b) failed to induce neutralizing antibodies. 15 Correct folding of the E protein may require the coordinated synthesis of the prM protein, since these proteins are found in heterodimers in the cell-associated forms of West Nile virus (Wengler et al., 1989a). The proper folding of E and the assembly of E and prM into viral particles may require 20 the coordinated synthesis of the NS1 protein, which is coretained in an early compartment of the secretory apparatus along with immature forms of E in JEV-infected cells (Mason, 1989).

Attempts to produce recombinant flavivirus 25 vaccines based on the flavivirus glycoproteins has met with some success, although protection in animal model systems has not always correlated with the predicted production of neutralizing antibodies (Bray et al., 1989; Deubel et al., 1988; Matsuura et al., 1989; Yasuda et al., 1990; Zhang et 30 al., 1988; Zhao et al., 1987).

Yasuda et al. (1990) reported a vaccinia recombinant containing the region of JEV encoding 65 out of the 127 amino acids of C, all of prM, all of E, and 59 out of the 352 amino acids of NS1. Haishi et al. (1989) 35 reported a vaccinia recombinant containing Japanese encephalitis sequences encoding 17 out of the 167 amino

acids of prM, all of E and 57 out of the 352 amino acids of NS1.

Deubel et al. (1988) reported a vaccinia recombinant containing the dengue-2 coding sequences for all 5 of C, all of prM, all of E and 16 out of the 352 amino acids of NS1.

Zhao et al. (1987) reported a vaccinia recombinant containing the dengue-4 coding sequences for all of C, all of prM, all of E, all of NS1, and all of NS2A. Bray et al. 10 (1989) reported a series of vaccinia recombinants containing the dengue-4 coding sequences for (i) all of C, all of prM and 416 out of the 454 amino acids of E, (ii) 15 out of the 167 amino acids of prM and 416 out of the 454 amino acids of E, (iii) 18 amino acids of influenza A virus hemagglutinin 15. and 416 out of the 454 amino acids of E, and (iv) 71 amino acids of respiratory syncytial virus G glycoprotein and 416 out of the 454 amino acids of E.

Despite these attempts to produce recombinant flavivirus vaccines, the proper expression of the JEV E 20 protein by the vaccinia recombinants has not been satisfactorily obtained. Although Haishi et al. (1989) demonstrated cytoplasmic expression of JEV E protein by their vaccinia recombinant, the distribution was different from that observed in JEV infected cells. Yasuda et al. 25 (1990) detected expression of JEV E protein by their vaccinia recombinant on the cell surface. Recombinant viruses that express the prM and E protein protected mice from approximately 10 LD₅₀ of challenge virus. Yasuda et al. (1990) elicited anti-JEV immune responses as well as 30 protection but reactivity to a panel of E specific monoclonal antibodies exhibited differences from the reactivity observed in JEV infected cells.

Dengue type 2 structural proteins have been expressed by recombinant vaccinia viruses (Deubel et al., 35 1988). Although these viruses induced the synthesis of the structural glycoprotein within infected cells, they neither elicited detectable anti-dengue immune responses nor

protected monkeys from dengue infection. Several studies also have been completed on the expression of portions of the dengue type 4 structural and nonstructural proteins in vaccinia virus (Bray et al., 1989; Falgout et al., 1989; 5 Zhao et al., 1987). Interestingly, a recombinant that contained the entire 5' end of the viral ORF extending from C to NS2A under the control of the P7.5 early- late promoter produced intracellular forms of prM, E, and NS1 but failed to induce the synthesis of extracellular forms of any of the 10 structural proteins, even though a form of NS1 was released from cells infected with this recombinant virus (Bray et al., 1989; Zhao et al., 1987). Additional recombinant viruses that contained several forms of the dengue type 4 E gene with or without other structural protein genes have 15 also been examined (Bray et al., 1989). Although several of these recombinant viruses were able to induce protection, they neither produced extracellular forms of E nor induced neutralizing antibodies. A dengue-vaccinia recombinant expressing a C-terminally truncated E protein gene induced 20 the synthesis of an extracellular form of E and provided an increasing level of resistance to dengue virus encephalitis in inoculated mice (Men et al., 1991).

It can thus be appreciated that provision of a flavivirus recombinant poxvirus which produces properly 25 processed forms of flavivirus proteins, and of vaccines which provide protective immunity against flavivirus infections, would be a highly desirable advance over the current state of technology.

OBJECTS OF THE INVENTION

30 It is therefore an object of this invention to provide recombinant poxviruses, which viruses express properly processed gene products of flavivirus, and to provide a method of making such recombinant poxviruses.

35 It is an additional object of this invention to provide for the cloning and expression of flavivirus coding sequences in a poxvirus vector.

It is another object of this invention to provide a vaccine which is capable of eliciting flavivirus neutralizing antibodies, hemagglutination-inhibiting antibodies and protective immunity against flavivirus infection and a lethal flavivirus challenge.

These and other objects and advantages of the present invention will become more readily apparent after consideration of the following.

STATEMENT OF THE INVENTION

In one aspect, the present invention relates to a recombinant poxvirus generating an extracellular flavivirus structural protein capable of inducing protective immunity against flavivirus infection. In particular, the recombinant poxvirus generates an extracellular particle containing flavivirus E and M proteins capable of eliciting neutralizing antibodies and hemagglutination-inhibiting antibodies. The poxvirus is advantageously a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus. The flavivirus is advantageously Japanese encephalitis virus, yellow fever virus and Dengue virus.

According to the present invention, the recombinant poxvirus contains therein DNA from flavivirus in a nonessential region of the poxvirus genome for expressing in a host flavivirus structural protein capable of release to an extracellular medium. In particular, the DNA contains Japanese encephalitis virus coding sequences that encode a precursor to structural protein M, structural protein E, and nonstructural proteins NS1 and NS2A. More in particular, the recombinant poxvirus contains therein DNA from flavivirus in a nonessential region of the poxvirus genome for expressing a particle containing flavivirus structural protein E and structural protein M.

In another aspect, the present invention relates to a vaccine for inducing an immunological response in a host animal inoculated with the vaccine, said vaccine including a carrier and a recombinant poxvirus containing, in a nonessential region thereof, DNA from flavivirus.

More in particular, the recombinant viruses express portions of the flavivirus ORF extending from prM to NS2B. Biochemical analysis of cells infected with the recombinant viruses showed that the recombinant viruses 5 specify the production of properly processed forms of all three flavivirus glycoproteins - prM, E, and NS1. The recombinant viruses induced synthesis of extracellular particles that contained fully processed forms of the M and E proteins. Furthermore, the results of mouse immunization 10 studies demonstrated that the induction of neutralizing antibodies and high levels of protection were associated with the ability of the immunizing recombinant viruses to produce extracellular particles containing the two structural membrane proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

A better understanding of the present invention will be had by referring to the accompanying drawings, in which:

20 FIG. 1 schematically shows a method for the construction of donor plasmids pSPJEVSH12VC and pSPJEVL14VC containing coding sequences for a portion of the JEV structural protein coding region, NS1 and NS2A;

25 FIG. 2 schematically shows a method for the construction of donor plasmids pSPJEV11VC and pSPJEV10VC containing coding sequences for a portion of the JEV structural protein coding region, NS1, NS2A and NS2B;

30 FIG. 3 shows the DNA sequence of oligonucleotides (shown with translational starts and stops in italics and early transcriptional stops underlined) used to construct the donor plasmids;

FIG. 4 is a map of the JEV coding regions inserted in the four recombinant vaccinia viruses VP650, VP555, VP658 and VP583;

35 FIG. 5 shows a comparison by SDS-PAGE analysis of the cell lysate NS1 proteins produced by JEV infection and infection with the recombinant vaccinia viruses VP650, VP555, VP658 and VP583;

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FIG. 6 shows a comparison by SDS-PAGE analysis of the culture fluid NS1 proteins produced by JEV infection and infection with the recombinant vaccinia viruses vP650, vP555, vP658 and vP583;

5 FIG. 7 shows a comparison by SDS-PAGE analysis of the cell lysate E proteins produced by JEV infection and infection with the recombinant vaccinia viruses vP650, vP555, vP658 and vP583;

10 FIG. 8 shows a comparison by SDS-PAGE analysis of the culture fluid E proteins produced by JEV infection and infection with the recombinant vaccinia viruses vP650, vP555, vP658 and vP583;

15 FIG. 9 shows a comparison by sucrose gradient analysis of the forms of the E protein found in the culture fluid harvested from JEV infected cells and cells infected with vaccinia recombinants vP555 and vP650;

20 FIG. 10 shows a comparison by immunoprecipitation analysis of the JEV-specific reactivity of the pre-challenge sera from animals vaccinated with JEV and with vaccinia recombinants vP555 and vP658;

FIG. 11 schematically shows a method for the construction of plasmid pSD460 for deletion of thymidine kinase gene and generation of recombinant vaccinia virus vP410;

25 FIG. 12 schematically shows a method for the construction of plasmid pSD486 for deletion of hemorrhagic region and generation of recombinant vaccinia virus vP553;

30 FIG. 13 schematically shows a method for the construction of plasmid pMP494 Δ for deletion of ATI region and generation of recombinant vaccinia virus vP618;

FIG. 14 schematically shows a method for the construction of plasmid pSD467 for deletion of hemagglutinin gene and generation of recombinant vaccinia virus vP723;

35 FIG. 15 schematically shows a method for the construction of plasmid pMPCSK1 Δ for deletion of gene cluster [C7L - K1L] and generation of recombinant vaccinia virus vP804;

FIG. 16 schematically shows a method for the construction of plasmid PSD548 for deletion of large subunit, ribonucleotide reductase and generation of recombinant vaccinia virus vP866 (NYVAC);

5 FIG. 17 shows the DNA sequence of the Nakayama strain of JEV in the region encoding C through NS2B;

FIG. 18 is a map of the JEV coding regions inserted in the vaccinia viruses vP555, vP825, vP908, vP923, vP857, vP864 and canarypox virus vCP107;

10 FIG. 19 is a map of the YF coding regions inserted in the vaccinia viruses vP766, vP764, vP869, vP729, vP725, vP984, vP997, vP1002, vP1003 and canarypox virus vCP127;

FIG. 20 shows part of the DNA sequence of a Western Pacific strain of DEN type 1;

15 FIG. 21 is a map of the DEN coding regions inserted in the vaccinia viruses vP867, vP962 and vP955.

FIG. 22 shows the DNA sequence of a canarypox PvuII fragment containing the C5 ORF;

20 FIG. 23 schematically shows a method for the construction of plasmid pRW848 for deletion of C5;

FIG. 24 shows the DNA sequence of a 7351 base pair fragment of canarypox containing the C3 ORF.

DETAILED DESCRIPTION OF THE INVENTION

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

Example 1 - CLONING OF JEV GENES INTO A VACCINIA VIRUS DONOR PLASMID

30 A thymidine kinase mutant of the Copenhagen strain of vaccinia virus, vP410 (Guo et al., 1989), was used to generate recombinant vP658 (see below). A recombinant vaccinia virus (vP425) containing the Beta-galactosidase gene in the HA region under the control of the 11-kDa late vaccinia virus promoter (Guo et al., 1989) was used to 35 generate recombinants vP555, vP583 and vP650. All vaccinia virus stocks were produced in either VERO (ATCC CCL81) or MRC-5 (ATCC CCL171) cells in Eagle's minimal essential medium (MEM) plus 10% heat-inactivated fetal bovine serum

(FBS). Biosynthetic studies were performed using baby hamster kidney cells (BHK 21-15 clone) grown at 37°C in MEM supplemented with 7.5% FBS and antibiotics, or VERO cells grown under the same conditions except using 5% FBS. The 5 JEV virus used in all *in vitro* experiments was a clarified culture fluid prepared from C6/36 cells infected with a passage 55 suckling mouse brain suspension of the Nakayama strain of JEV (Mason, 1989).

Restriction enzymes were obtained from GIBCO/BRL, 10 Inc., (Gaithersburg, MD), New England BioLabs, Inc. (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN). T4 DNA ligase was obtained from New England BioLabs, Inc. Standard recombinant DNA techniques were used (Maniatis et al., 1986) with minor modifications 15 for cloning, screening, and plasmid purification. Nucleic acid sequences were confirmed using standard dideoxy chain-termination reactions (Sanger et al., 1977) on alkaline-denatured double-stranded plasmid templates. Sequencing primers, and other oligonucleotides were 20 synthesized using standard chemistries (Biosearch 8700, San Rafael, CA; Applied Biosystems 380B, Foster City, CA). The JEV cDNAs used to construct the JEV-vaccinia recombinant viruses were derived from the Nakayama strain of JEV (McAda et al., 1987); all nucleotide coordinates are derived from 25 the sequence data presented in FIG. 17A and B (SEQ ID NO:52) which contains the sequence of the C coding region combined with an updated sequence of prM, E, NS1, NS2A and NS2B coding regions.

Plasmid pJEV3/4 was derived by cloning a 30 BglII-ApaI fragment of JEV cDNA (nucleotides 2554-3558), an ApaI-BamI fragment (nucleotides 3559-4125), and annealed oligos J3 (SEQ ID NO:44) and J4 (SEQ ID NO:45) [FIG. 3; containing a translation stop followed by a vaccinia early transcription termination signal (TTTTTAT; Yuen et al., 35 1987), an EaqI site, and a HindIII sticky end] into BamHI-HindIII digested pUC18. pJEV3/4 was digested within the JEV sequence by EcoRV (nucleotide 2672) and within pUC18

by SacI, and the fragment containing the plasmid origin and JEV cDNA sequences extending from nucleotides 2672-4125 was ligated to a SacI-EcoRV fragment of JEV cDNA (nucleotides 2125-2671). The resulting plasmid, pJEV1, contained the 5 viral ORF extending from the SacI site (nucleotide 2125) in the last third of E through the BalI site (nucleotide 4125) two amino acid residues (aa) into the predicted N terminus of NS2B (FIG. 1).

Synthetic oligos J1B (SEQ ID NO:46) and J2B (SEQ 10. ID NO:47) (FIG. 3; containing a XhoI sticky end, a SmaI site, the last 15 aa of C, and first 9 aa of JEV prM with a sticky HindIII end) were ligated to a HindIII-SacI fragment of JEV cDNA (nucleotides 407-2124), and XhoI-SacI digested vector pIBI24 (International Biotechnologies Inc., New 15 Haven, CT). The resulting plasmid, pJEV2, contained the viral ORF extending between the methionine (Met) codon (nucleotides 337-339) occurring 15 aa preceding the predicted N terminus of prM and the SacI site (nucleotide 2124) found in the last third of E (FIG. 1).

Synthetic oligos J7 (SEQ ID NO:48) and J8 (SEQ ID 20. NO:49) (FIG. 3; containing BamHI and NcoI sticky ends) were used to clone the NcoI-SacI fragment of JEV cDNA (nucleotides 1336-2124) into BamHI-SacI digested pIBI24 yielding pSPNC78. Oligonucleotides J9 (SEQ ID NO:50) and 25 J10 (SEQ ID NO:51) (FIG. 3; containing a HindIII sticky end, a SmaI site, and nucleotides 811-832 of JEV cDNA) were used to clone a HincII-NcoI fragment of JEV cDNA (nucleotides 833-1335) into HindIII-NcoI digested pSPNC78. The resulting plasmid, pJEV5, contained the viral ORF extending between 30 the Met codon (nucleotides 811-813) occurring 25 aa preceding the N terminus of E and the SacI site (nucleotide 2124) found in the last third of E (FIG. 1).

pTP15 contains the early/late vaccinia virus H6 promoter inserted into a polylinker region flanked by 35 sequences from the HindIII A fragment of vaccinia virus from which the hemagglutinin (HA) gene has been deleted (Guo et al., 1989). SmaI-EagI digested pTP15 was purified and

ligated to the purified SmaI-SacI insert from pJEV2 plus the SacI-EagI insert of pJEV1, yielding pSPJEVL (FIG. 1). The 6 bp corresponding to the unique SmaI site used to produce pSPJEVL were then removed using
5 oligonucleotide-directed double-strand break mutagenesis (Mandecki, 1986), creating pSPJEVL14VC in which the H6 promoter immediately preceded the ATG start codon (FIG. 1).

The SmaI-EagI pTP15 fragment was ligated to the purified SmaI-SacI insert from pJEV5 plus the SacI-EagI 10 insert of pJEV1, yielding pSPJEVSH (FIG. 1). The 6 bp corresponding to the unique SmaI site used to produce pSPJEVSH were removed as described above, creating pSPJEVSH12VC in which the H6 promoter immediately preceded the ATG start codon (FIG. 1).

15 Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to change a potential vaccinia virus early transcription termination signal (Yuen et al., 1987) in the E gene of pJEV2 (TTTTGT; nucleotides 1304-1310) to TCTTTGT, creating plasmid pJEV22 (FIG. 2). The same change was performed on pJEV5 producing pJEV6 (FIG. 2).

20 Synthetic oligos J37 and J38 [FIG. 3; containing JEV nucleotides 4497-4512, a translation stop, an early transcription termination signal (TTTTTAT; Yuen et al., 1987), an EagI site, and HindIII sticky end] were used to clone a SacI-DraI fragment of JEV cDNA (nucleotides 2125-4496) into SacI-HindIII digested pIBI24. The resulting plasmid, pJEV7, contained the viral ORF extending between the SacI site (nucleotide 2125) found in the last third of E and the last codon of NS2B (nucleotide 4512) (FIG. 2).
25 SmaI-EagI digested pTP15 was purified and ligated to the purified SmaI-SacI insert from pJEV22 plus the SacI-EagI insert of pJEV7, yielding pSPJEV10 (FIG. 2). The 6 bp corresponding to the SmaI site used to create pSPJEV10 were removed as described above, creating pSPJEV10VC (FIG. 2).
30 SmaI-EagI digested pTP15 was purified and ligated to the purified SmaI-SacI insert from pJEV6 plus the SacI-EagI insert of pJEV7, yielding pSPJEV11 (FIG. 2). The 6 bp corresponding to the SmaI site used to create pSPJEV11 were removed as described above, creating pSPJEV11VC (FIG. 2).
35 Ligation of the SmaI-EagI digested pTP15 with the SmaI-SacI insert of pJEV6 and SacI-EagI insert of pJEV7 yielded pSPJEV11 (FIG. 2). The 6 bp corresponding to the SmaI site

used to create pSPJEV11 were removed as described above, yielding pSPJEV11VC (FIG. 2).

Example 2 - CONSTRUCTION OF VACCINIA VIRUS RECOMBINANTS

Procedures for transfection of recombinant donor plasmids into tissue culture cells infected with a rescuing vaccinia virus and identification of recombinants by *in situ* hybridization on nitrocellulose filters have been described (Guo et al., 1989; Panicali et al., 1982). pSPJEVL14VC, pSPJEVSH12VC, and pSPJEV10VC were transfected into VP425-infected cells to generate the vaccinia recombinants VP555, VP583 and VP650, respectively (FIG. 4). pSPJEV11VC was transfected into VP410 infected cells to generate the vaccinia recombinant VP658 (FIG. 4).

Example 3 - IN VITRO VIRUS INFECTION AND RADIOLABELING

BHK or VERO cell monolayers were prepared in 35 mm diameter dishes and infected with vaccinia viruses (m.o.i. of 2) or JEV (m.o.i. of 5) and incubated for 11 hr (vaccinia) or 16 hr (JEV) before radiolabeling. At 11 hr or 16 hr post-infection, the medium was removed and replaced with warm Met-free medium containing 2% FBS and 250 μ Ci/ml of 35 S-Met. The cells were incubated for 1 hr at 37°C, rinsed with warm maintenance medium containing 10-times the normal amount of unlabeled Met, and incubated in this same high Met medium 6 hr before harvesting as described below.

In some cases, samples of clarified culture fluid were analyzed by sucrose gradient centrifugation in 10 to 35% continuous sucrose gradients prepared, centrifuged, and analyzed as described (Mason, 1989).

Example 4 - RADIOIMMUNOPRECIPITATIONS, POLYACRYLAMIDE GEL ELECTROPHORESIS, AND ENDOGLYCOSIDASE TREATMENT

Radiolabeled cell lysates and culture fluids were harvested and the viral proteins were immunoprecipitated, digested with endoglycosidases, and separated in SDS-containing polyacrylamide gels (SDS-PAGE) exactly as described (Mason, 1989). Unless otherwise noted, all SDS-PAGE samples were prepared by heating in the presence of 50 mM dithiothreitol (DTT) before electrophoresis.

Example 5 - STRUCTURE OF RECOMBINANT VACCINIA VIRUSES

Four different vaccinia virus recombinants were constructed that expressed portions of the JEV coding region extending from prM through NS2B. The JEV cDNA sequences contained in these recombinant viruses are shown in FIG. 4. In all four recombinant viruses the sense strand of the JEV cDNA was positioned behind the vaccinia virus early/late H6 promoter, and translation was expected to be initiated from naturally occurring JEV Met codons located at the 5' ends of the viral cDNA sequences (FIG. 4).

Recombinant vP555 encodes the putative 15 aa signal sequence preceding the N terminus of the structural protein precursor prM, the structural glycoprotein E, the nonstructural glycoprotein NS1, and the nonstructural protein NS2A (McAda et al., 1987). Recombinant vP583 encodes the putative signal sequence preceding the N terminus of E, E, NS1, and NS2A (McAda et al., 1987). Recombinant vP650 contains a cDNA encoding the same proteins as vP555 with the addition of the NS2B coding region. Recombinant vP658 contains a cDNA encoding the same proteins as vP583 with the addition of NS2B. In recombinants vP650 and vP658, a potential vaccinia virus early transcription termination signal in E (TTTTTGT; nucleotides 1087-1094) was modified to TCTTTGT without altering the aa sequence. This change was made in an attempt to increase the level of expression of E and NS1, since this sequence has been shown to increase transcription termination in *in vitro* transcription assays (Yuen et al., 1987).

The location and orientation of the JEV genes within the recombinant vaccinia genomes were confirmed by restriction enzyme digestion of recombinant vaccinia virus DNA. During these analyses it was noted that recombinants vP555, vP583, and vP650 had a deletion from within the HindIII C fragment through HindIII N and M and into HindIII K. This same deletion was observed in the vP425 parental virus. Interestingly, these viruses were less cytopathic in VERO cells than vP410 and its derivative vP658.

NS1 was Properly Processed and Secreted when Expressed by Recombinant Vaccinia Viruses

FIGS. 5 and 6 show a comparison of the NS1 proteins produced by JEV infection or infection with the recombinant vaccinia viruses. BHK cells were infected with JEV or recombinant vaccinia viruses, then labeled for 1 hr with ³⁵S-Met, and chased for 6 hr. Equal fractions of the cell lysate (FIG. 5) or culture fluid (FIG. 6) prepared from each cell layer were immunoprecipitated, and then either mock digested (M), digested with endo H (H), or digested with PNGase F (F), prior to SDS-PAGE analysis.

The data from the pulse-chase experiments depicted in FIGS. 5 and 6 demonstrate that proteins identical in size to authentic NS1 and NS1' were synthesized in and secreted from cells infected with any of the 4 recombinant vaccinia viruses. Furthermore, the sensitivity of these proteins to endo H and PNGase F indicated that the recombinant forms of NS1 were glycosylated. Specifically, the cell-associated forms of NS1 all contained two immature (endo H sensitive) N-linked glycans, while the extracellular forms contained one immature and one complex or hybrid (endo H resistant) glycan (see Mason, 1989). Interestingly, these pulse-chase studies showed similar levels of NS1 production by all four recombinants, suggesting that the potential vaccinia early transcriptional termination signal present near the end of the E coding region in vP555 and vP583 did not significantly reduce the amount of NS1 produced relative to vP650 or vP658 in which the TTTTGTT was modified. Although the experiments depicted in FIGS. 5 and 6 were conducted on BHK cells 11 hr post-infection, similar experiments with infected VERO cells pulse-labeled at 4 or 8 hr post-infection did not reveal any differences in NS1 expression associated with the presence or absence of this TTTTGTT sequence. Comparison of the synthesis of NS1 from vaccinia viruses containing either the NS2A (vP555 and vP583) or both the NS2A and NS2B (vP650 and vP658) coding regions showed that the presence or absence of the NS2B coding region had no affect on NS1 expression. These results are consistent with the results of Falgout et

al. (1989) showing that only the NS2A gene is needed for the proper processing of NS1.

E and prM were Properly Processed when Expressed by Recombinant Vaccinia Viruses

FIGS. 7 and 8 show a comparison of the E protein produced by JEV infection or infection with the recombinant vaccinia viruses. BHK cells were infected with JEV or recombinant vaccinia viruses, then labeled for 1 hr with ³⁵S-Met, and chased for 6 hr. Equal fractions of the cell lysate (FIG. 7) or culture fluid (FIG. 8) prepared from each cell layer were immunoprecipitated, and then either mock digested (M), digested with endo H (H), or digested with PNGase F (F), prior to SDS-PAGE analysis.

The data from the pulse-chase experiments depicted in FIGS. 7 and 8 demonstrate that proteins identical in size to E were synthesized in cells infected with all recombinant vaccinia viruses containing the E gene. However, the E protein was only released from cells infected with vaccinia viruses that contained the region of the viral ORF encoding prM, E, NS1, and NS2A (vP555 and vP650; see FIGS. 4, 7 and 8). Endoglycosidase sensitivity (FIGS. 7 and 8) revealed that both the intracellular and extracellular forms of the E protein synthesized by cells infected with the vaccinia recombinants were glycosylated; the cell-associated forms of E were endo H sensitive, whereas the extracellular forms were resistant to endo H digestion.

Immunoprecipitates prepared from radiolabeled vaccinia-infected cells using a MAb specific for M (and prM) revealed that prM was synthesized in cells infected with vP555 and vP650. Cells infected with either of these recombinant vaccinia viruses produced cellular forms of prM that were identical in size to the prM protein produced by JEV-infected cells, and a M protein of the correct size was detected in the culture fluid of cells infected with these two viruses.

The extracellular fluid harvested from cells infected with vP555 and vP650 contained forms of E that migrated with a peak of hemagglutinating activity in sucrose

density gradients. Interestingly, this hemagglutinin migrated similarly to the slowly sedimenting peak of noninfectious hemagglutinin (SHA) (Russell et al., 1980) found in the culture fluid of JEV-infected cells (FIG. 9).

5 Furthermore, these same fractions contained the fully processed form of M, demonstrating that vP555- and vP650-infected cells produced a particle that contained both of the structural membrane proteins of JEV. These particles probably represent empty JEV envelopes, analogous to the 22

10 nm hepatitis B virus particles found in the blood of humans infected with hepatitis B virus (Tiollais et al., 1985), and released from cells expressing the hepatitis B surface antigen gene (Dubois et al., 1980; Moriarty et al., 1981). The hemagglutinating properties of the supernatant fluid of

15 cells infected with the recombinant viruses was examined, since hemagglutination activity requires particulate forms of JEV proteins that are sensitive to disruption by detergents (Eckels et al., 1975). These hemagglutination assays showed that the supernatant fluids harvested from

20 cells infected with vP555 and vP650 contained hemagglutinating activity that was inhibited by anti-JEV antibodies and had a pH optimum identical to the JEV hemagglutinin. No hemagglutinating activity was detected in the culture fluid of cells infected with vP410, vP583, or

25 vP658.

Recombinant Vaccinia Viruses Generate Extracellular Particles

Recombinant vaccinia virus vP555 produced E- and M-containing extracellular particles that behaved like empty viral envelopes. The ability of this recombinant virus to induce the synthesis of extracellular particles containing the JEV structural proteins provides a system to generate properly processed and folded forms of these antigens.

The recombinant viruses described herein contain portions of the JEV ORF that encode the precursor to the structural protein M, the structural protein E, and nonstructural proteins NS1, NS2A, and NS2B. The E and NS1 proteins produced by cells infected with these recombinant

viruses underwent proteolytic cleavage and N-linked carbohydrate addition in a manner indistinguishable from the same proteins produced by cells infected with JEV. These data further demonstrate that the proteolytic cleavage and 5 N-linked carbohydrate addition to E and NS1 do not require flavivirus nonstructural proteins located 3' to NS2A in the viral genome (Bray et al., 1989; Deubel et al., 1988; Falgout et al., 1989; Fan et al., 1990; Matsuura et al., 1989; Ruiz-Linares et al., 1989; Yasuda et al., 1990; Zhang 10 et al., 1988; Zhao et al., 1987).

Interestingly, the portion of the ORF inserted in the recombinant vaccinia viruses had a significant effect on the late-stage processing of prM and E, but not on the fate of NS1. All recombinant viruses that encoded NS1 produced 15 mature extracellular forms of this protein, consistent with previous studies showing that NS1 produced in the presence of NS2A and NS2B was properly processed and secreted from transfected cells (Fan et al., 1990). On the other hand, only two of the four recombinants that contained the E 20 protein coding region produced extracellular forms of E. These two recombinants, VP555 and VP650, differed from the remaining recombinants in that they contained the prM coding region in addition to E, NS1, and NS2A. The findings that 25 extracellular forms of E were produced only by viruses containing the coding regions for both E and prM and that the extracellular forms of E were associated with M suggest that the simultaneous synthesis of prM and E is a requirement for the formation of particles that are targeted for the extracellular fluid.

30 Example 6 - ANIMAL PROTECTION STUDIES

Groups of 3-week-old outbred Swiss mice were immunized by intraperitoneal injection with 10^7 pfu of vaccinia virus diluted in 0.1 ml of PBS. Three weeks after inoculation, selected mice were bled from the retroorbital 35 sinus, and sera were stored at -70°C. Two to three days after bleeding, the mice were either re-inoculated with the recombinant virus or challenged by intraperitoneal injection

with dilutions of suckling mouse brain infected with JEV (Beijing strain; multiple mouse passage) (Huang, 1982). Due to the variations in lethal dose observed between groups of mice and passages of the challenge virus, lethal-dose titrations were performed in each challenge experiment. Following challenge, mice were observed at daily intervals for three weeks.

Evaluation of Immune Response to the Recombinant Vaccinia Viruses

Pools of mouse sera were prepared by mixing equal aliquots of sera from the representative animals bled in each group. Three-microliter samples of pooled sera were mixed with detergent-treated cell culture fluid obtained from ³⁵S-Met-labeled JEV-infected cells, and the antigen antibody mixtures were then incubated with fixed *Staphylococcus aureus* bacteria (The Enzyme Center, Malden, MA) that were coated with rabbit anti-mouse immunoglobulins (Dakopatts, Gostrup, Denmark) to assure that all classes of murine antibodies would be precipitated. The samples obtained from these precipitations were not treated with dithiothreitol prior to electrophoresis in order to avoid electrophoretic artifacts that resulted from the co-migration of the rabbit immunoglobulin heavy chain with the radiolabeled viral antigens, and to permit clear separation of the E and the NS1' proteins. Neutralization tests were performed on heat-inactivated sera (20 min. at 56°C) as described (Tesh et al., 1987) with the following modifications: (1) freshly thawed human serum was added to all virus/antibody dilutions to a final concentration of 2.5%, (2) following virus absorption, the cell monolayers were overlayed with medium containing 0.5% carboxymethylcellulose (Sigma, St. Louis, MO), and (3) plaques were visualized at 6 days post-infection by staining with 0.1% crystal violet dissolved in 20% ethanol. Hemagglutination tests and hemagglutination-inhibition (HAI) tests were performed by a modification of the method of Clarke et al. (1958).

Vaccination with vP555 Provided Protection Against Greater than 10,000 LD₅₀ of JEV

The recombinant vaccinia viruses were tested for their ability to protect outbred mice from lethal JEV infection using the Beijing strain of JEV, which exhibits high peripheral pathogenicity in mice (Huang, 1982). Based on preliminary experiments which showed that all four recombinant vaccinia viruses could provide some protection from a lethal challenge of this virus, two viruses (vP555 and vP658) were selected for in-depth challenge studies.

vP555 induced the synthesis of extracellular forms of E, whereas vP658 did not produce any extracellular forms of E, but contained additional cDNA sequences encoding the NS2B protein. In the challenge experiments several dilutions of challenge virus were tested, the effect of a booster immunization with vaccinia recombinants on the levels of protection was examined, and the serological responses in a subset of the vaccinated animals were evaluated. The results of a single inoculation with these recombinant viruses showed that recombinant virus vP555 produced better levels of protection than vP658 at all challenge doses (Table 1). Both recombinant viruses provided better protection at lower levels of challenge virus, consistent with the ability to overwhelm protection with high doses of JEV. Table 1 also shows that complete protection from more than 10,000 LD₅₀ of JEV was achieved by two inoculations with vP555, which was not the case for vP658 at the challenge doses tested. FIG. 10 shows an analysis of the JEV-specific reactivity of pre-challenge sera from animals vaccinated with the recombinant vaccinia viruses. Sera collected from a subset of the animals used in the protection experiments (see Tables 1 and 2) were pooled and aliquots were tested for their ability to immunoprecipitate radiolabeled proteins harvested from the culture fluid of JEV-infected cells. The two lanes on the right side of the autoradiogram of FIG. 10 were prepared from samples immunoprecipitated with sera obtained from uninoculated mice (-) or from a mouse that survived a normally lethal dose of

JEV. The analysis demonstrated that: (1) only those animals immunized with vP555 showed a strong immune response to E, and (2) a second inoculation resulted in a significant increase in reactivity to the E protein (FIG. 10).

5 Analysis of the neutralization and HAI data for the sera collected from these animals confirmed the results of the immunoprecipitation analyses, showing that the animals boosted with vP555, which were 100% protected, had very high levels of neutralizing and
10 hemagglutination-inhibiting antibodies (Table 2). These levels of neutralizing and hemagglutination-inhibiting antibodies were similar to the titers achieved in naive mice that survived challenge from a normally lethal dose of the Beijing strain of JEV.

15 The ability of vP555 to induce neutralizing antibodies may be related to the fact that vP555 produces an extracellular particulate form of the structural proteins E and M. This SHA-like particle probably represents an empty JEV envelope that contains E and M folded and assembled into
20 a configuration very similar to that found in the infectious JEV particle. Recombinants vP555 and vP650 may generate extracellular forms of the structural proteins because they contain the coding regions for all three JEV glycoproteins, thereby providing all of the JEV gene products needed for
25 assembly of viral envelopes. Other investigators (see above) have not been able to detect the production of extracellular forms of E by cells expressing all three structural proteins (C, prM, and E) in the presence or absence of NS1 and NS2A. The inability of their recombinant
30 viruses to produce particles similar to those produced by vP555 and vP650 could be due to the presence of the C protein gene in their recombinant genomes. In particular, it is possible that the C protein produced in the absence of a genomic RNA interferes with the proper assembly of the
35 viral membrane proteins. Alternatively, an incompletely processed form of C similar to that detected by Nowak et al. (1989) in in vitro translation experiments, could prevent

release of the structural membrane proteins from the cells expressing the C gene.

Table 1. Evaluation of ability of recombinant vaccinia virus vP555 or vP658 to protect mice from fatal JEV encephalitis.

	IMMUNIZING VIRUS ¹	CHALLENGE DOSE (LOG) ²	SURVIVAL AFTER ONE INOCULATION ³	SURVIVAL AFTER TWO INOCULATIONS ⁴
10	vP410	-1	0/20	0/10
	vP410	-2	0/20	1/10
	vP410	-3	0/18	
15	vP555	-1	12/20	10/10
	vP555	-2	15/20	10/10
	vP555	-3	18/19	
20	vP658	-1	0/20	3/9
	vP658	-2	4/22	3/10
	vP658	-3	12/18	
25	-	-2	0/5	1/5
	-	-3	1/10	3/5
	-	-4	2/10	4/10
	-	-5	3/10	6/10
	-	-6	4/10	3/10
	-	-7	3/5	7/10
	-	-8		5/6

30 ¹ Vaccinia recombinant used for immunization, or unimmunized lethal dose titration groups (-).

35 ² Dilution of suckling mouse brain stock delivered in the challenge. Based on the simultaneous titration data shown in this table, the challenge dose of -1 log of virus was equivalent to 4.7×10^4 LD₅₀ for the 6-week-old animals challenged following one inoculation, and 3.0×10^4 LD₅₀ for the 10-week-old animals challenged following two inoculations.

40 ³ Live animals/total for each group; challenge delivered to 6-week-old mice, three weeks following a single inoculation.

45 ⁴ Live animals/total for each group; challenge delivered to 10-week-old mice, 6 weeks following the first vaccinia inoculation and 3 weeks following a second inoculation with the same vaccinia recombinant.

Table 2. Plaque reduction neutralization titers and HAI antibody titers in pre-challenge sera.

5	GROUP ¹	<u>ONE INOCULATION</u>		<u>TWO INOCULATIONS</u>		HAI ³ TITER
		NEUTRALIZATION ² TITER	HAI ³ TITER	NEUTRALIZATION ² TITER	HAI ³ TITER	
	vP410 GROUP 1	<1:10	<1:10			
	vP555 GROUP 1	1:40	1:40			
10	vP555 GROUP 2	1:80	1:160	1:640		1:160
	VP658 GROUP 1	<1:10	<1:10			
	VP658 GROUP 2	<1:10	<1:10	<1:10		<1:10

1 Vaccinia recombinant used for immunization. Group 1 indicates animals challenged 3 weeks following a single vaccinia inoculation, and group 2 indicates animals challenged following two inoculations.

2 Serum dilution yielding 90% reduction in plaque number.

3 Serum dilution.

Example 7 - ATTENUATED VACCINIA VACCINE STRAIN NYVAC

To develop a new vaccinia vaccine strain, NYVAC (vP866), the Copenhagen vaccine strain of vaccinia virus was modified by the deletion of six nonessential regions of the genome encoding known or potential virulence factors. The sequential deletions are detailed below. All designations of vaccinia restriction fragments, open reading frames and nucleotide positions are based on the terminology reported in Goebel et al., 1990a,b.

The deletion loci were also engineered as recipient loci for the insertion of foreign genes.

The regions sequentially deleted in NYVAC are listed below. Also listed are the abbreviations and open reading frame designations for the deleted regions (Goebel et al., 1990a,b) and the designation of the vaccinia recombinant (vP) containing all deletions through the deletion specified:

- 40 (1) thymidine kinase gene (TK; J2R) vP410;
- (2) hemorrhagic region (u; B13R + B14R) vP553;
- (3) A type inclusion body region (ATI; A26L) vP618;
- (4) hemagglutinin gene (HA; A56R) vP723;
- (5) host range gene region (C7L - K1L) vP804; and

(6) large subunit, ribonucleotide reductase (I4L) vP866
(NYVAC).

DNA Cloning and Synthesis

Plasmids were constructed, screened and grown by standard procedures (Maniatis et al., 1986; Perkus et al., 1985; Piccini et al., 1987). Restriction endonucleases were obtained from GIBCO/BRL, Gaithersburg, MD, New England Biolabs, Beverly, MA; and Boehringer Mannheim Biochemicals, Indianapolis, IN. Klenow fragment of *E. coli* polymerase was obtained from Boehringer Mannheim Biochemicals. BAL-31 exonuclease and phage T4 DNA ligase were obtained from New England Biolabs. The reagents were used as specified by the various suppliers.

Synthetic oligodeoxyribonucleotides were prepared on a Biosearch 8750 or Applied Biosystems 380B DNA synthesizer as previously described (Perkus et al., 1989). DNA sequencing was performed by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase (Tabor et al., 1987) as previously described (Guo et al., 1989). DNA amplification by polymerase chain reaction (PCR) for sequence verification (Engelke et al., 1988) was performed using custom synthesized oligonucleotide primers and GeneAmp DNA amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT) in an automated Perkin Elmer Cetus DNA Thermal Cycler. Excess DNA sequences were deleted from plasmids by restriction endonuclease digestion followed by limited digestion by BAL-31 exonuclease and mutagenesis (Mandecki, 1986), using synthetic oligonucleotides.

Cells, Virus, and Transfection

The origins and conditions of cultivation of the Copenhagen strain of vaccinia virus has been previously described (Guo et al., 1989). Generation of recombinant virus by recombination, *in situ* hybridization of nitrocellulose filters and screening for Beta-galactosidase activity are as previously described (Panicali et al., 1982; Perkus et al., 1989).

Construction of Plasmid pSD460 for Deletion of Thymidine Kinase Gene (J2R)

Referring now to FIG. 11, plasmid pSD406 contains vaccinia HindIII J (pos. 83359 - 88377) cloned into pUC8. 5 pSD406 was cut with HindIII and PvuII, and the 1.7 kb fragment from the left side of HindIII J cloned into pUC8 cut with HindIII/SmaI, forming pSD447. pSD447 contains the entire gene for J2R (pos. 83855 - 84385). The initiation codon is contained within an NlaIII site and the termination 10 codon is contained within an SspI site. Direction of transcription is indicated by an arrow in FIG. 11.

To obtain a left flanking arm, a 0.8 kb HindIII/EcoRI fragment was isolated from pSD447, then digested with NlaIII and a 0.5 kb HindIII/NlaIII fragment 15 isolated. Annealed synthetic oligonucleotides MPSYN43/MPSYN44 (SEQ ID NO:1/SEQ ID NO:2)

MPSYN43	$\begin{matrix} 5' & \text{TAATTAACTAGCTACCCGGG} \\ & \text{NlaIII} \end{matrix}$	3'
MPSYN44	$\begin{matrix} 3' & \text{GTACATTAATTGATCGATGGGCCCTTAA} \\ & \text{EcoRI} \end{matrix}$	5'

20 were ligated with the 0.5 kb HindIII/NlaIII fragment into pUC18 vector plasmid cut with HindIII/EcoRI, generating plasmid pSD449.

To obtain a restriction fragment containing a 25 vaccinia right flanking arm and pUC vector sequences, pSD447 was cut with SspI (partial) within vaccinia sequences and HindIII at the pUC/vaccinia junction, and a 2.9 kb vector fragment isolated. This vector fragment was ligated with annealed synthetic oligonucleotides MPSYN45/MPSYN46 (SEQ ID NO:3/SEQ ID NO:4).

MPSYN45	$\begin{matrix} 5' & \text{AGCTTCCCGGGTAAGTAATACTACGTCAAGGAGAAAACGAA} \\ & \text{HindIII SmaI} \end{matrix}$	3'
MPSYN46	$\begin{matrix} 3' & \text{AGGGCCCATTCATTATGCAGTTCCCTTTGCTT} \\ & \text{SspI} \end{matrix}$	5'

35 ACGATCTGTAGTTAGCGGGCCGCTAATTAACATAAT 3' MPSYN45
 TGCTAGACATCAATCGCCGGGGATTAATTGATTA 5' MPSYN46
 generating pSD459.

40 To combine the left and right flanking arms into one plasmid, a 0.5 kb HindIII/SmaI fragment was isolated from pSD449 and ligated with pSD459 vector plasmid cut with

HindIII/SmaI, generating plasmid PSD460. PSD460 was used as donor plasmid for recombination with wild type parental vaccinia virus Copenhagen strain VC-2. 32 P labeled probe was synthesized by primer extension using MPSYN45 (SEQ ID NO:3) as template and the complementary 20mer oligonucleotide MPSYN47 (SEQ ID NO:5) (5' TTAGTTAATTAGGCGGCCGC 3') as primer. Recombinant virus VP410 was identified by plaque hybridization.

10 Construction of Plasmid PSD486 for Deletion of Hemorrhagic Region (B13R + B14R)

Referring now to FIG. 12, plasmid PSD419 contains vaccinia SalI G (pos. 160,744-173,351) cloned into pUC8. PSD422 contains the contiguous vaccinia SalI fragment to the right, SalI J (pos. 173,351-182,746) cloned into pUC8. To construct a plasmid deleted for the hemorrhagic region, γ , B13R - B14R (pos. 172,549 - 173,552), PSD419 was used as the source for the left flanking arm and PSD422 was used as the source of the right flanking arm. The direction of transcription for the γ region is indicated by an arrow in FIG. 12.

To remove unwanted sequences from PSD419, sequences to the left of the NcoI site (pos. 172,253) were removed by digestion of PSD419 with NcoI/SmaI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation generating plasmid PSD476. A vaccinia right flanking arm was obtained by digestion of PSD422 with HpaI at the termination codon of B14R and by digestion with NruI 0.3 kb to the right. This 0.3 kb fragment was isolated and ligated with a 3.4 kb HincII vector fragment isolated from PSD476, generating plasmid PSD477. The location of the partial deletion of the vaccinia γ region in PSD477 is indicated by a triangle. The remaining B13R coding sequences in PSD477 were removed by digestion with ClaI/HpaI, and the resulting vector fragment was ligated

with annealed synthetic oligonucleotides SD22mer/SD20mer
(SEQ ID NO:6/SEQ ID NO:7)

5 ClaI BamHI HpaI
SD22mer 5' CGATTACTATGAAGGATCCGTT 3'
SD20mer 3' TAATGATATTCCTAGGCAA 5'

generating pSD479. pSD479 contains an initiation codon (underlined) followed by a BamHI site. To place *E. coli* Beta-galactosidase in the B13-B14 (u) deletion locus under 10 the control of the u promoter, a 3.2 kb BamHI fragment containing the Beta-galactosidase gene (Shapira et al., 1983) was inserted into the BamHI site of pSD479, generating pSD479BG. pSD479BG was used as donor plasmid for recombination with vaccinia virus vP410. Recombinant 15 vaccinia virus vP533 was isolated as a blue plaque in the presence of chromogenic substrate X-gal. In vP533 the B13R-B14R region is deleted and is replaced by Beta-galactosidase.

20 To remove Beta-galactosidase sequences from vP533, plasmid pSD486, a derivative of pSD477 containing a polylinker region but no initiation codon at the u deletion junction, was utilized. First the ClaI/HpaI vector fragment from pSD477 referred to above was ligated with annealed synthetic oligonucleotides SD42mer/SD40mer (SEQ ID NO:8/SEQ 25 ID NO:9)

25 ClaI SacI XbaI HpaI
SD42mer 5' CGATTACTAGATCTGAGCTCCCCGGCTCGAGGGATCCGTT 3'
SD40mer 3' TAATGATCTAGACTCGAGGGGCCGAGCTCCCTAGGCAA 5'
 BglII SmaI BamHI

30 generating plasmid pSD478. Next the EcoRI site at the pUC/vaccinia junction was destroyed by digestion of pSD478 with EcoRI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation, generating plasmid pSD478E⁻. pSD478E⁻ was digested with BamHI and HpaI and 35 ligated with annealed synthetic oligonucleotides HEM5/HEM6 (SEQ ID NO:10/SEQ ID NO:11)

BamHI EcoRI HpaI
HEM5 5' GATCCGAATTCTAGCT 3'
HEM6 3' GCTTAAGATCGA 5'

generating plasmid pSD486. pSD486 was used as donor plasmid for recombination with recombinant vaccinia virus vP533, generating vP553, which was isolated as a clear plaque in the presence of X-gal.

5 Construction of Plasmid pMP494Δ for Deletion of ATI Region (A26L)

Referring now to FIG. 13, pSD414 contains SalI B cloned into pUC8. To remove unwanted DNA sequences to the left of the A26L region, pSD414 was cut with XbaI within 10 vaccinia sequences (pos. 137,079) and with HindIII at the pUC/vaccinia junction, then blunt ended with Klenow fragment of *E. coli* polymerase and ligated, resulting in plasmid pSD483. To remove unwanted vaccinia DNA sequences to the right of the A26L region, pSD483 was cut with EcoRI (pos. 15 140,665 and at the pUC/vaccinia junction) and ligated, forming plasmid pSD484. To remove the A26L coding region, pSD484 was cut with NdeI (partial) slightly upstream from the A26L ORF (pos. 139,004) and with HpaI (pos. 137,889) slightly downstream from the A26L ORF. The 5.2 kb vector 20 fragment was isolated and ligated with annealed synthetic oligonucleotides ATI3/ATI4 (SEQ ID NO:12/SEQ ID NO:13).

NdeI

ATI3 5' TATGAGTAACCTAACCTTTGTTAATTAAAAGTATATTCAAAAAATAAGT
ATI4 3' ACTCATTGAATTGAGAAAACAATTAAATTTCATATAAGTTTTTATTCA

25

BglII EcoRI HpaI

TATATAAATAGATCTGAATTGTT 3' ATI3
ATATATTTATCTAGACTTAAGCAA 5' ATI4

reconstructing the region upstream from A26L and replacing 30 the A26L ORF with a short polylinker region containing the restriction sites BglII, EcoRI and HpaI, as indicated above. The resulting plasmid was designated pSD485. Since the BglII and EcoRI sites in the polylinker region of pSD485 are not unique, unwanted BglII and EcoRI sites were removed from 35 plasmid pSD483 (described above) by digestion with BglII (pos. 140,136) and with EcoRI at the pUC/vaccinia junction, followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation. The resulting plasmid was 40 designated pSD489. The 1.8 kb ClaI (pos. 137,198)/EcoRV (pos. 139,048) fragment from pSD489 containing the A26L ORF

was replaced with the corresponding 0.7 kb polylinker-containing ClaI/EcoRV fragment from pSD485, generating pSD492. The BglII and EcoRI sites in the polylinker region of pSD492 are unique.

5 A 3.3 kb BglII cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Perkus et al., 1990) was inserted into the BglII site of pSD492, forming pSD493KBG. Plasmid pSD493KBG was used in recombination with rescuing virus vP553. Recombinant 10 vaccinia virus, vP581, containing Beta-galactosidase in the A26L deletion region, was isolated as a blue plaque in the presence of X-gal.

To generate a plasmid for the removal of Beta-15 galactosidase sequences from vaccinia recombinant virus vP581, the polylinker region of plasmid pSD492 was deleted by mutagenesis (Mandecki, 1986) using synthetic oligonucleotide MPSYN177 (SEQ ID NO:14)

(5' AAAATGGCGTGGATTGTTAACCTTATATAACTTATTTTTGAATATAC 3').

20 In the resulting plasmid, pMP494Δ, vaccinia DNA encompassing positions [137,889 - 138,937], including the entire A26L ORF is deleted. Recombination between the pMP494Δ and the Beta-galactosidase containing vaccinia recombinant, vP581, resulted in vaccinia deletion mutant vP618, which was 25 isolated as a clear plaque in the presence of X-gal.

Construction of Plasmid pSD467 for Deletion of Hemagglutinin Gene (A56R)

Referring now to FIG. 14, vaccinia SalI G restriction fragment (pos. 160,744-173,351) crosses the 30 HindIII A/B junction (pos. 162,539). pSD419 contains vaccinia SalI G cloned into pUC8. The direction of transcription for the hemagglutinin (HA) gene is indicated by an arrow in FIG. 14. Vaccinia sequences derived from HindIII B were removed by digestion of pSD419 with HindIII within vaccinia sequences and at the pUC/vaccinia junction followed by ligation. The resulting plasmid, pSD456, contains the HA gene, A56R, flanked by 0.4 kb of vaccinia sequences to the left and 0.4 kb of vaccinia sequences to

the right. A56R coding sequences were removed by cutting pSD456 with RsaI (partial; pos. 161,090) upstream from A56R coding sequences, and with EagI (pos. 162,054) near the end of the gene. The 3.6 kb RsaI/EagI vector fragment from 5 pSD456 was isolated and ligated with annealed synthetic oligonucleotides MPSYN59 (SEQ ID NO:15), MPSYN62 (SEQ ID NO:16), MPSYN60 (SEQ ID NO:17), and MPSYN 61 (SEQ ID NO:18)

RsaI
 MPSYN59 5' ACACGAATGATTCTAAAGTATTGGAAAGTTTATAGGTAGTT-
 MPSYN62 3' TGTGCTTACTAAAAGATTCAAAACCTTCAAAATATCCATCAA-
 MPSYN59 GATAGAACAAAATACATAATT 3'
 MPSYN62 CTATCT 5'

15 MPSYN60 5' TGTAATAAATCACTTTTATACTAAGATC-
 MPSYN61 3' TGTTTATGTATTAAACATTTTATTAGTAAAAATATGATTCTAG- BglII

20 MPSYN60 -SmaI PstI EagI -TCCCGGGCTGCAGC 3'
 MPSYN61 -AGGGCCCGACGTCGCCGG 5'

reconstructing the DNA sequences upstream from the A56R ORF and replacing the A56R ORF with a polylinker region as indicated above. The resulting plasmid is pSD466. The vaccinia deletion in pSD466 encompasses positions [161,185- 25 162,053]. The site of the deletion in pSD466 is indicated by a triangle in FIG. 14.

A 3.2 kb BglII/BamHI (partial) cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Guo et al., 1989) was inserted into the BglII site of pSD466, forming pSD466KBG. Plasmid pSD466KBG was used in recombination with rescuing virus vP618. Recombinant vaccinia virus, vP708, containing Beta-galactosidase in the A56R deletion, was isolated as a blue 35 plaque in the presence of X-gal.

Beta-galactosidase sequences were deleted from vP708 using donor plasmid pSD467. pSD467 is identical to pSD466, except that EcoRI, SmaI and BamHI sites were removed from the pUC/vaccinia junction by digestion of pSD466 with 40 EcoRI/BamHI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation. Recombination between

vP708 and pSD467 resulted in recombinant vaccinia deletion mutant, vP723, which was isolated as a clear plaque in the presence of X-gal.

5 Construction of Plasmid pMPCSK1Δ for Deletion of Open
Reading Frames [C7L-K1L]

Referring now to FIG. 15, the following vaccinia clones were utilized in the construction of pMPCSK1Δ. pSD420 is SalI H cloned into pUC8. pSD435 is KpnI F cloned into pUC18. pSD435 was cut with SphI and religated, forming 10 pSD451. In pSD451, DNA sequences to the left of the SphI site (pos. 27,416) in HindIII M are removed (Perkus et al., 1990). pSD409 is HindIII M cloned into pUC8.

To provide a substrate for the deletion of the [C7L-K1L] gene cluster from vaccinia, *E. coli* Beta-galactosidase was first inserted into the vaccinia M2L deletion locus (Guo et al., 1990) as follows. To eliminate the BglII site in pSD409, the plasmid was cut with BglII in 15 vaccinia sequences (pos. 28,212) and with BamHI at the pUC/vaccinia junction, then ligated to form plasmid pMP409B. pMP409B was cut at the unique SphI site (pos. 27,416). M2L 20 coding sequences were removed by mutagenesis (Guo et al., 1990; Mandecki, 1986) using synthetic oligonucleotide

25 BglII
MPSYN82 (SEQ ID NO:19) 5' TTTCTGTATATTGCACCAATTAGATCTTACTC
AAAATATGTAACAAATA 3'

The resulting plasmid, pMP409D, contains a unique BglII site inserted into the M2L deletion locus as indicated above. A 3.2 kb BamHI (partial)/BglII cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the 30 control of the 11 kDa promoter (Bertholet et al., 1985) was inserted into pMP409D cut with BglII. The resulting plasmid, pMP409DBG (Guo et al., 1990), was used as donor plasmid for recombination with rescuing vaccinia virus vP723. Recombinant vaccinia virus, vP784, containing Beta-galactosidase inserted into the M2L deletion locus, was 35 isolated as a blue plaque in the presence of X-gal.

A plasmid deleted for vaccinia genes [C7L-K1L] was assembled in pUC8 cut with SmaI, HindIII and blunt ended

with Klenow fragment of *E. coli* polymerase. The left flanking arm consisting of vaccinia HindIII C sequences was obtained by digestion of pSD420 with XbaI (pos. 18,628) followed by blunt ending with Klenow fragment of *E. coli* 5 polymerase and digestion with BglII (pos. 19,706). The right flanking arm consisting of vaccinia HindIII K sequences was obtained by digestion of pSD451 with BglII (pos. 29,062) and EcoRV (pos. 29,778). The resulting plasmid, pMP581CK is deleted for vaccinia sequences between 10 the BglII site (pos. 19,706) in HindIII C and the BglII site (pos. 29,062) in HindIII K. The site of the deletion of vaccinia sequences in plasmid pMP581CK is indicated by a triangle in FIG. 15.

To remove excess DNA at the vaccinia deletion junction, plasmid pMP581CK, was cut at the NcoI sites within vaccinia sequences (pos. 18,811; 19,655), treated with Bal-31 exonuclease and subjected to mutagenesis (Mandecki, 1986) using synthetic oligonucleotide MPSYN233 (SEQ ID NO:20) 5' TGTCATTTAACACTATACTCATATTAAATAAATATTATTATT 3'.

20 The resulting plasmid, pMPCSK1Δ, is deleted for vaccinia sequences positions 18,805-29,108, encompassing 12 vaccinia open reading frames [C7L - K1L]. Recombination between pMPCSK1Δ and the Beta-galactosidase containing vaccinia recombinant, vP784, resulted in vaccinia deletion mutant, 25 vP804, which was isolated as a clear plaque in the presence of X-gal.

Construction of Plasmid pSD548 for Deletion of Large Subunit, Ribonucleotide Reductase (I4L)

Referring now to FIG. 16, plasmid pSD405 contains 30 vaccinia HindIII I (pos. 63,875-70,367) cloned in pUC8. pSD405 was digested with EcoRV within vaccinia sequences (pos. 67,933) and with SmaI at the pUC/vaccinia junction, and ligated, forming plasmid pSD518. pSD518 was used as the source of all the vaccinia restriction fragments used in the 35 construction of pSD548.

The vaccinia I4L gene extends from position 67,371-65,059. Direction of transcription for I4L is indicated by an arrow in FIG. 16. To obtain a vector

plasmid fragment deleted for a portion of the I4L coding sequences, pSD518 was digested with BamHI (pos. 65,381) and HpaI (pos. 67,001) and blunt ended using Klenow fragment of *E. coli* polymerase. This 4.8 kb vector fragment was ligated 5 with a 3.2 kb SmaI cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Perkus et al., 1990), resulting in plasmid pSD524KBG. pSD524KBG was used as donor plasmid for recombination with 10 vaccinia virus vP804. Recombinant vaccinia virus, vP855, containing Beta-galactosidase in a partial deletion of the I4L gene, was isolated as a blue plaque in the presence of X-gal.

To delete Beta-galactosidase and the remainder of 15 the I4L ORF from vP855, deletion plasmid pSD548 was constructed. The left and right vaccinia flanking arms were assembled separately in pUC8 as detailed below and presented schematically in FIG. 16.

To construct a vector plasmid to accept the left 20 vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and ligated with annealed synthetic oligonucleotides 518A1/518A2 (SEQ ID NO:21/SEQ ID NO:22).

BamHI RsaI
518A1 5' GATCCTGAGTACTTGAAATATAATGATATATTTCACTTATCTCAT
25 518A2 3' GACTCATGAAACATTATATTACTATATAAAAAGTGAATAGAGTA

BglII EcoRI
TTGAGAATAAAAAGATCTTAGG 3' 518A1
AACTCTTATTTCTAGAACCTTAA 5' 518A2

30 forming plasmid pSD531. pSD531 was cut with RsaI (partial) and BamHI and a 2.7 kb vector fragment isolated. pSD518 was cut with BglII (pos. 64,459)/ RsaI (pos. 64,994) and a 0.5 kb fragment isolated. The two fragments were ligated together, forming pSD537, which contains the complete 35 vaccinia flanking arm left of the I4L coding sequences.

To construct a vector plasmid to accept the right vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and ligated with annealed synthetic oligonucleotides 518B1/518B2 (SEQ ID NO:23/SEQ ID NO:24)

518B1 5' GATCCAGATCTCCGGGAAAAAAATTATTTAACCTTCATTAATAGGG
518B2 3' GTCTAGAGGGCCCTTTTTAATAAAATTGAAAAGTAATTATCCC

5 RsaI EcoRI
 ATTTGACGTATGTAGCGTACTAGG 3' 518B1
 TAAACTGCATACTACGCATGATCCTTAA 5' 518B2

forming plasmid pSD532. pSD532 was cut with RsaI (partial)/EcoRI and a 2.7 kb vector fragment isolated.

10 pSD518 was cut with RsaI within vaccinia sequences (pos. 67,436) and EcoRI at the vaccinia/pUC junction, and a 0.6 kb fragment isolated. The two fragments were ligated together, forming pSD538, which contains the complete vaccinia flanking arm to the right of I4L coding sequences.

15 The right vaccinia flanking arm was isolated as a 0.6 kb EcoRI/BglII fragment from pSD538 and ligated into pSD537 vector plasmid cut with EcoRI/BglII. In the resulting plasmid, pSD539, the I4L ORF (pos. 65,047-67,386) is replaced by a polylinker region, which is flanked by 0.6
20 kb vaccinia DNA to the left and 0.6 kb vaccinia DNA to the right, all in a pUC background. The site of deletion within vaccinia sequences is indicated by a triangle in FIG. 16. To avoid possible recombination of Beta-galactosidase sequences in the pUC-derived portion of pSD539 with Beta-
25 galactosidase sequences in recombinant vaccinia virus vP855, the vaccinia I4L deletion cassette was moved from pSD539 into pRC11, a pUC derivative from which all Beta-galactosidase sequences have been removed and replaced with a polylinker region (Colinas et al., 1990). pSD539 was cut
30 with EcoRI/PstI and the 1.2 kb fragment isolated. This fragment was ligated into pRC11 cut with EcoRI/PstI (2.35 kb), forming pSD548. Recombination between pSD548 and the Beta-galactosidase containing vaccinia recombinant, vP855, resulted in vaccinia deletion mutant vP866, which was
35 isolated as a clear plaque in the presence of X-gal.

DNA from recombinant vaccinia virus vP866 was analyzed by restriction digests followed by electrophoresis on an agarose gel. The restriction patterns were as expected. Polymerase chain reactions (PCR) (Engelke et al.,

1988) using vP866 as template and primers flanking the six deletion loci detailed above produced DNA fragments of the expected sizes. Sequence analysis of the PCR generated fragments around the areas of the deletion junctions 5 confirmed that the junctions were as expected. Recombinant vaccinia virus vP866, containing the six engineered deletions as described above, was designated vaccinia vaccine strain "NYVAC."

10. Example 8 - CONSTRUCTION OF NYVAC-MV RECOMBINANT EXPRESSING MEASLES FUSION AND HEMAGGLUTININ GLYCOPROTEINS

CDNA copies of the sequences encoding the HA and F proteins of measles virus MV (Edmonston strain) were inserted into NYVAC to create a double recombinant 15 designated NYVAC-MV. The recombinant authentically expressed both measles glycoproteins on the surface of infected cells. Immunoprecipitation analysis demonstrated correct processing of both F and HA glycoproteins. The recombinant was also shown to induce syncytia formation.

20. Cells and Viruses

The rescuing virus used in the production of NYVAC-MV was the modified Copenhagen strain of vaccinia virus designated NYVAC. All viruses were grown and titered on Vero cell monolayers.

25. Plasmid Construction

Plasmid pSPM2LHA (Taylor et al., 1991) contains the entire measles HA gene linked in a precise ATG to ATG configuration with the vaccinia virus H6 promoter which has been previously described (Taylor et al., 1988a,b; Guo et al., 1989; Perkus et al., 1989). A 1.8 kbp EcoRV/SmaI fragment containing the 3' most 24 bp of the H6 promoter fused in a precise ATG:ATG configuration with the HA gene lacking the 3' most 26 bp was isolated from pSPM2LHA. This fragment was used to replace the 1.8 kbp EcoRV/SmaI fragment of pSPMHHA11 (Taylor et al., 1991) to generate pRW803. Plasmid pRW803 contains the entire H6 promoter linked precisely to the entire measles HA gene.

In the confirmation of previous constructs with the measles HA gene it was noted that the sequence for codon 18 (CCC) was deleted as compared to the published sequence (Alkhatib et al., 1986). The CCC sequence was replaced by 5 oligonucleotide mutagenesis via the Kunkel method (Kunkel, 1985) using oligonucleotide RW117 (SEQ ID NO:39) (5' GACTATCCTACTTCCCTTGGGATGGGGTTATCTTGTA-3').

PRO 18

Single stranded template was derived from plasmid pRW819 which contains the H6/HA cassette from pRW803 in pIBI25 (International Biotechnologies, Inc., New Haven, CT). The mutagenized plasmid containing the inserted (CCC) to encode for a proline residue at codon 18 was designated pRW820. The sequence between the HindIII and XbaI sites of pRW820 was confirmed by nucleotide sequence analysis. The HindIII site is situated at the 5' border of the H6 promoter while the XbaI site is located 230 bp downstream from the initiation codon of the HA gene. A 1.6 kbp XbaI/EcoRI fragment from pRW803, containing the HA coding sequences downstream from the XbaI (above) and including the termination codon, was used to replace the equivalent fragment of pRW820 resulting in the generation of pRW837. The mutagenized expression cassette contained within pRW837 was derived by digestion with HindIII and EcoRI, blunt-ended 25 using the Klenow fragment of *E. coli* DNA polymerase in the presence of 2mM dNTPs, and inserted into the SmaI site of pSD513 to yield pRW843. Plasmid pSD513 was derived from plasmid pSD460 by the addition of polylinker sequences. Plasmid pSD460 was derived to enable deletion of the 30 thymidine kinase gene from vaccinia virus (FIG. 11).

To insert the measles virus F gene into the HA insertion plasmid, manipulations were performed on pSPHMF7. Plasmid pSPHMF7 (Taylor et al., 1991) contains the measles F gene juxtaposed 3' to the previously described vaccinia 35 virus H6 promoter. In order to attain a perfect ATG for ATG configuration and remove intervening sequences between the 3' end of the promoter and the ATG of the measles F gene

oligonucleotide directed mutagenesis was performed using oligonucleotide SPMAD (SEQ ID NO:40).

SPMAD: 5'- TATCCGTTAAGTTGTATCGTAATGGGTCTCAAGGTGAACGTCT-3'

The resultant plasmid was designated pSPMF75M20.

- 5 The plasmid pSPMF75M20 which contains the measles F gene now linked in a precise ATG for ATG configuration with the H6 promoter was digested with NruI and EacI. The resulting 1.7 kbp blunt ended fragment containing the 3' most 27 bp of the H6 promoter and the entire fusion gene was
- 10 isolated and inserted into an intermediate plasmid pRW823 which had been digested with NruI and XbaI and blunt ended. The resultant plasmid pRW841 contains the H6 promoter linked to the measles F gene in the pIBI25 plasmid vector (International Biotechnologies, Inc., New Haven, CT). The
- 15 H6/measles F cassette was excised from pRW841 by digestion with SmaI and the resulting 1.8 kb fragment was inserted into pRW843 (containing the measles HA gene). Plasmid pRW843 was first digested with NotI and blunt-ended with Klenow fragment of *E. coli* DNA polymerase in the presence of
- 20 2mM dNTPs. The resulting plasmid, pRW857, therefore contains the measles virus F and HA genes linked in a tail to tail configuration. Both genes are linked to the vaccinia virus H6 promoter.

Development of NYVAC-MV

- 25 Plasmid pRW857 was transfected into NYVAC infected Vero cells by using the calcium phosphate precipitation method previously described (Panicali et al., 1982; Piccini et al., 1987). Positive plaques were selected on the basis of *in situ* plaque hybridization to specific MV F and HA radiolabeled probes and subjected to 6 sequential rounds of plaque purification until a pure population was achieved. One representative plaque was then amplified and the resulting recombinant was designated NYVAC-MV (vP913).
- 30
- 35 Example 9 - CLONING OF JEV GENES INTO A VACCINIA VIRUS DONOR PLASMID

A thymidine kinase mutant of the Copenhagen strain of vaccinia virus vP410 (Guo et al., 1989) was used to generate recombinants vP825, vP829, vP857 and vP864 (see

below). The generation of vP555 has previously been described (Mason et al., 1991). All vaccinia virus stocks were produced in VERO (ATCC CCL81) cells in Eagle's minimal essential medium plus 10% heat inactivated fetal bovine serum (FBS). Biosynthetic studies were performed using VERO Cells grown at 37°C in MEM supplemented with 5% FBS and antibiotics, or HeLa (ATCC CCL2) cells grown under the same conditions except using 10% FBS and non-essential amino acids. The JEV virus used in all *in vitro* experiments was a clarified culture fluid prepared from C6/36 cells infected with a passage 55 suckling mouse brain suspension of the Nakayama strain of JEV (Mason, 1989). Animal challenge experiments were performed using the highly pathogenic P3 strain of JEV (multiple mouse passage; Huang, 1982).

CDNA encoding the C protein of JEV was obtained by a modification of the method of Okayama and Berg (1982) using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL, Gaithersburg, MD) (D'Alessio and Gerrard, 1988). Genomic RNA was isolated from virions prepared by the method of Repik et al. (1983) from suspension cultures of C6/36 cells infected with a passage 55 suckling mouse brain stock of the Nakayama strain of JEV. First strand cDNA synthesis was primed from a synthetic oligonucleotide complementary to bases 986 to 1005 of the E coding region of JEV (FIG. 17A and B) (SEQ ID NO:52). The double-stranded cDNA was ligated to synthetic oligonucleotides containing the EcoRI site (New England Biolabs, Beverly, MA), inserted into phosphatase treated EcoRI-cleaved pBR322 (New England Biolabs), and the resulting DNA was used to transform *E. coli* strain DH5 cells (GIBCO/BRL). Plasmids were analyzed by restriction enzyme digestion and a plasmid (pc20) containing cDNA corresponding to 81 nucleotides of non-coding RNA and the C and prM coding regions was identified. pc20 was digested at the linker sites with EcoRI and at an internal DraI site situated 28 bp 5' of the ATG initiation codon and the resulting fragment containing the C and prM coding regions was inserted into SmaI-EcoRI digested pUC18, creating plasmid, pDr20. The

sequence of the C coding region of pc20, combined with an updated sequence of the prM, E, NS1, NS2A, and NS2B coding regions of the Nakayama strain of JEV is presented in FIG. 17A and B (SEQ ID NO:52). All nucleotide coordinates are based on this updated sequence with numbering beginning at the C protein Met initiation codon.

Plasmid pDr20 containing JEV cDNA (nucleotides -28 to 1000) in the SmaI and EcoRI sites of pUC18 (see above) was digested with BamH I and EcoRI and the JEV cDNA insert cloned into pIBI25 (International Biotechnologies, Inc., New Haven, CT) generating plasmid JEV18. JEV18 was digested with ApaI within the JE sequence (nucleotide 24) and XbaI within pIBI25 and ligated to annealed oligonucleotides J90 (SEQ ID NO:54) and J91 (SEQ ID NO:55) (containing an XbaI sticky end, SmaI site, and JE nucleotides 1 to 23) generating plasmid JEV19. JEV19 was digested with XbaI within pIBI25 and AccI within JE sequences (nucleotide 602) and the resulting 613 bp fragment was cloned into the XbaI and AccI fragment of JEV2 (FIG. 1) containing the plasmid origin and JEV cDNA encoding the carboxy-terminal 40% prM and amino-terminal two thirds of E (nucleotides 603 to 2124), generating plasmid JEV20 containing JE sequences from the ATG of C through the SacI site (nucleotide 2124) found in the last third of E.

The SmaI-SacI fragment from JEV8 (a plasmid analogous to JEV1 (FIG. 1) in which TTTTGT nucleotides 1304 to 1310 were changed to TCTTG), containing JE sequences from the last third of E through the first two amino acids of NS2B (nucleotides 2124 to 4126), the plasmid origin and vaccinia sequences, was ligated to the purified SmaI-SacI insert from JEV20 yielding JEV22-1. The 6 bp corresponding to the unique SmaI site used to construct JEV22-1 were removed using oligonucleotide-directed double-strand break mutagenesis (Mandecki, 1986) creating JEV24 in which the H6 promoter immediately preceded the ATG start codon.

Plasmid JEV7 (FIG. 2) was digested with SphI within JE sequences (nucleotide 2381) and HindIII within

IBI24. Ligation to annealed oligonucleotides J94 and J95 [containing a SphI sticky end, translation stop, a vaccinia early transcription termination signal (TTTTTAT; Yuen et al., 1987) a translation stop, an EagI site and a HindIII sticky end] generated plasmid JEV25 which contains JE cDNA extending from the SacI site (nucleotide 2124) in the last third of E through the carboxy-terminus of E. The SacI-EagI fragment from JEV25 was ligated to the SacI-EagI fragment of JEV8 (containing JE cDNA encoding 15 aa C, prM and amino-terminal two thirds of E nucleotides 337 to 2124, the plasmid origin and vaccinia sequences) yielding plasmid JEV26. A unique SmaI site preceding the ATG start codon was removed as described above, creating JEV27 in which the H6 promoter immediately preceded the ATG start codon.

15 Oligonucleotides J96, J97, J98 and J99 (containing JE nucleotides 2293 to 2380 with an SphI sticky end) were kinased, annealed and ligated to SmaI-SphI digested and alkaline phosphatase treated pIBI25 generating plasmid JEV28. JEV28 was digested with HpaI within the JE sequence 20 (nucleotide 2301) and with HindIII within the pIBI25 sequence and alkaline phosphatase treated. Ligation to the HpaI-HindIII fragment from JEV1 or HpaI-HindIII fragment from JEV7 (FIG. 2) yielded JEV29 [containing a SmaI site followed by JE cDNA encoding 30 aa E, NS1, NS2A (nucleotides 2293 to 4125)] and JEV30 [containing a SmaI site followed by JE cDNA encoding 30 aa E, NS1, NS2A, NS2B (nucleotides 2293 to 4512)].

30 The SmaI-EagI fragment from JEV29 was ligated to SmaI-EagI digested pTP15 (Mason et al., 1991) yielding JEV31. The 6 bp corresponding to the unique SmaI site used to produce JEV31 were removed as described above creating JEV33 in which the H6 promoter immediately preceded the ATG start codon.

35 The SmaI-EagI fragment from JEV30 was ligated to SmaI-EagI digested pTP15 yielding JEV32. The 6 bp corresponding to the unique SmaI site used to produce JEV32 were removed as described above creating JEV34 in which the

H6 promoter immediately preceded the ATG start codon.

Oligonucleotides J90 (SEQ ID NO:25), J91 (SEQ ID NO:26), J94 (SEQ ID NO:27), J95 (SEQ ID NO:28), J96 and J97 (SEQ ID NO:29), and J99 and J98 (SEQ ID NO:30) are as follows:

5 J90 5'-TCGAG CCCGGG atg ACTAAAAAACCAGGA GGGCC-3'
J91 3'- C GGGCCC TAC TGATTTTTGGTCCT C -5'
XbaI SmaI Apal

10 J94 5'- C T tga ttttat tga CGGCCG A -3'
 10 J95 3'-GTACG A ACT AAAATA ACT GCCGGC TTCGA-5'
SphI EagI HindIII

J96+J97 5'-GGG atg GGCGTTAACGACGAGACCGATCAATTGCTTGGCTTC
 J99+J98 3'-CCC TAC CCGCAATTGCGTGCTCTGGCTAGTTAACGGAAACCCGAAG

15 TTAGCCACAGGAGGTGTGCTCGTGTCTTAGCGACCAA
AATCGGTGTCTCCACACGAGCACAAGAATCGCTGGTT

Construction of Vaccinia Virus Recombinants

Procedures for transfection of recombinant donor plasmids into tissue culture cells infected with a rescuing vaccinia virus and identification of recombinants by *in situ* hybridization on nitrocellulose filters have been described (Panicali et al., 1982; Guo et al., 1989). JEV24, JEV27, JEV33 and JEV34 were transfected into vP410 infected cells to generate the vaccinia recombinants vP825, vP829, vP857 and vP864 respectively (FIG. 18).

In Vitro Virus Infection and Radiolabeling

HeLa cell monolayers were prepared in 35 mm diameter dishes and infected with vaccinia viruses (m.o.i. of 2) or JEV (m.o.i. of 5) before radiolabeling. At 16 h post infection, cells were pulse labeled with medium containing ^{35}S -Met and chased for 6 hr in the presence of excess unlabeled Met exactly as described by Mason et al. (1991). JEV-infected cells were radiolabeled as above for preparation of radioactive proteins for checking pre- and post-challenge mouse sera by radioimmunoprecipitation.

Radiimmunoprecipitations, Polyacrylamide Gel Electrophoresis, and Endoglycosidase Treatment

Radiolabeled cell lysates and culture fluids were harvested and the viral proteins were immunoprecipitated, digested with endoglycosidases, and separated in SDS-containing polyacrylamide gels (SDS-PAGE) exactly as described by Mason (1989).

Animal Protection Experiments

Mouse protection experiments were performed exactly as described by Mason et al. (1991). Briefly, groups of 3-week-old mice were immunized by intraperitoneal (ip) injection with 10^7 pfu of vaccinia virus, and 3 weeks later sera were collected from selected mice. Mice were then either re-inoculated with the recombinant virus or challenged by ip injection with a suspension of suckling mouse brain infected with the P3 strain of JEV. Three weeks later, the boosted animals were re-bled and challenged with the P3 strain of JEV. Following challenge, mice were observed at daily intervals for three weeks and lethal-dose titrations were performed in each challenge experiment using litter-mates of the experimental animals. In addition, sera were collected from all surviving animals 4 weeks after challenge.

Evaluation of Immune Response to the Recombinant Vaccinia Viruses

Sera were tested for their ability to precipitate JEV proteins from detergent-treated cell lysates or culture fluids obtained from 35 S-Met-labeled JEV-infected cells exactly as described by Mason et al. (1991).

Hemagglutination inhibition (HAI) and neutralization (NEUT) tests were performed as described by Mason et al. (1991) except 1% carboxymethylcellulose was used in the overlay medium and 5 day incubation was used for visualization of plaques for the NEUT test.

Structure of Recombinant Vaccinia Viruses

Four different vaccinia recombinants (in the HA locus) were constructed that expressed portions of the JEV coding region extending from C through NS2B. The JEV cDNA

sequences contained in these recombinant viruses are shown in FIG. 18. In all four recombinant viruses the sense strand of the JEV cDNA was positioned behind the vaccinia virus early/late H6 promoter, and translation was expected 5 to be initiated from naturally occurring JEV Met codons located at the 5' ends of the viral cDNA sequences.

Recombinant vP825 encoded the capsid protein C, structural protein precursor prM, the structural glycoprotein E, the nonstructural glycoprotein NS1, and the 10 nonstructural protein NS2A (McAda et al., 1987).

Recombinant vP829 encoded the putative 15 aa signal sequence preceding the amino-terminus of prM, as well as prM, and E (McAda et al., 1987). Recombinant vP857 contained a cDNA encoding the 30 aa hydrophobic carboxy-terminus of E, 15 followed by NS1 and NS2A. Recombinant vP864 contained a cDNA encoding the same proteins as vP857 with the addition of NS2B. In recombinants vP825 and vP829 a potential vaccinia virus early transcription termination signal in E (TTTTTGT; nucleotides 1399-1405) was modified to TCTTTGT without altering the aa sequence. This change was made in 20 an attempt to increase the level of expression of E since this sequence has been shown to increase transcription termination in *in vitro* transcription assays (Yuen et al., 1987).

25 E and prM Were Properly Processed When Expressed By Recombinant Vaccinia Viruses

Pulse-chase experiments demonstrate that proteins identical in size to E were synthesized in cells infected with all recombinant vaccinia viruses containing the E gene 30 (Table 3). In the case of cells infected with JEV, vP555 and vP829, an E protein that migrated slower in SDS-PAGE was also detected in the culture fluid harvested from the infected cells (Table 3). This extracellular form of E produced by JEV- and vP555-infected cells contained mature 35 N-linked glycans (Mason, 1989; Mason et al., 1991), as confirmed for the extracellular forms of E produced by vP829-infected cells. Interestingly, vP825, which contained the C coding region in addition to prM and E specified the

synthesis of E in a form that is not released into the extracellular fluid (Table 3). Immunoprecipitations prepared from radiolabeled vaccinia-infected cells using a MAb specific for M (and prM) revealed that prM was synthesized in cells infected with vP555, vP825, and vP829, and M was detected in the culture fluid of cells infected with vP555 or vP829 (Table 3).

The extracellular fluid harvested from cells infected with vP555 and vP829 contained an HA activity that was not detected in the culture fluid of cells infected with vP410, vP825, vP857 or vP864. The HA activity observed in the culture fluid of vP829 infected cells was 8 times as high as that obtained from vP555 infected cells. This HA appeared similar to the HA produced in JEV infected cells based on its inhibition by anti-JEV antibodies and its pH optimum (Mason et al., 1991). Analysis of sucrose density gradients prepared with culture fluids obtained from infected cells identified a peak of HA activity in the vP829 sample that co-migrated with the peak of slowly sedimented hemagglutinin (SHA) found in the JEV culture fluids (Table 3). This result indicated that vP829 infected cells produced extracellular particles similar to the empty viral envelopes containing E and M which are observed in the culture fluids harvested from vP555 infected cells (FIG. 9).

25 NS1 Was Properly Processed and Secreted When Expressed By Recombinant Vaccinia Virus

The results of pulse-chase experiments demonstrated that proteins identical in size to authentic NS1 and NS1' were synthesized in cells infected with vP555, vP825, vP857 and vP864 (Table 3). NS1 produced by vP555-infected cells was released into the culture fluid of infected cells in a higher molecular weight form. NS1 was also released into the culture fluid of cells infected with vP857 and vP864 (Table 3). Comparison of the synthesis of NS1 from vaccinia viruses containing either the NS2A (vP857) or both the NS2A and NS2B (vP864) coding regions showed that the presence or absence of the NS2B coding region had no affect on NS1 expression, consistent with previous data.

showing that only the NS2A gene is needed for the proper processing of NS1 (Falgout et al., 1989; Mason et al., 1991). The efficiency of release of NS1 by vP825 infected cells was more than 10 times less than that for NS1 synthesized in vP555, vP857 or vP864 infected cells.

Recombinant Vaccinia Viruses Induced Immune Responses To JEV Antigens

Pre-challenge sera pooled from selected animals in each group were tested for their ability to 10 immunoprecipitate radiolabeled E and NS1. The results of these studies (Table 3) demonstrated that: (1) the following order of immune response to E vP829>vP555>vP825, (2) all viruses encoding NS1 and NS2A induced antibodies to NS1, and (3) all immune responses were increased by a second 15 inoculation with the recombinant viruses. Analysis of the neutralization and HAI data for the sera collected from these animals (Table 4) confirmed the results of the immunoprecipitation analyses, showing that the immune response to E as demonstrated by RIP correlated well with 20 these other serological tests (Table 4).

Vaccination With the Recombinant Viruses Provided Protection From Lethal JEV Infection

All of the recombinant vaccinia viruses were able to provide mice with some protection from lethal infection 25 by the peripherally pathogenic P3 strain of JEV (Huang, 1982) (Table 4). These studies confirmed the protective potential of vP555 (Mason et al., 1991) and demonstrated similar protection in animals inoculated with vP825 and vP829. Recombinant viruses vP857 and vP864 which induced 30 strong immune responses to NS1 showed much lower levels of protection, but mice inoculated with these recombinants were still significantly protected when compared to mice inoculated with the control virus, vP410 (Table 4).

Post-Challenge Immune Responses Document the Level of JEV Replication

In order to obtain a better understanding of the mechanism of protection from lethal challenge in animals inoculated with these recombinant viruses, the ability of

antibodies in post-challenge sera to recognize JEV antigens was evaluated. For these studies an antigen from radiolabeled JEV-infected cell lysates was utilized and the response to the NS3 protein which induces high levels of antibodies in hyperimmunized mice (Mason et al., 1987a) was examined. The results of these studies (Table 5) correlated perfectly with the survival data in that groups of animals vaccinated with recombinant viruses that induced high levels of protection (vP829, vP555, and vP825) showed low post-challenge responses to NS3, whereas the sera from survivors of groups vaccinated with recombinants that expressed NS1 alone (vP857 and vP864) showed much higher post-challenge responses to NS3.

15 Table 3. Characterization of proteins expressed by vaccinia recombinants and their immune responses

	vP555-	vP829	vP825	vP857	vP864	
20						
	Proteins expressed					
25	Intracellular NS1	prM, E NS1	prM, E	prM, E NS1	NS1	NS1
	secreted	M, E, NS1	M, E	NS1	NS1	NS1
30	Particle formation	+	+	-	-	-
	Immune response single	E	E	NS1	NS1	NS1
35	double	E, NS1	E	E, NS1	NS1	NS1

40 single = single inoculation with 10^7 pfu vaccinia recombinants (ip)

double = two inoculations with 10^7 pfu vaccinia recombinants (ip) 3 weeks apart

Table 4. Protection of mice and immune response

Protection	vP555	vP829	vP825	vP857	vP864
single	7/10	10/10	8/10	0/10	1/10
double	10/10	9/10	9/10	5/10	6/10

Neut titer

single	1:20	1:160	1:10	<1:10	<1:10
double	1:320	1:2560	1:320	<1:10	<1:10

HAI titer

single	1:20	1:40	1:10	<1:10	<1:10
double	1:80	1:160	1:40	<1:10	<1:10

single = single inoculation with 10^7 pfu vaccinia recombinants (ip) and challenge 3 weeks later with 4.9×10^5 LD₅₀ P3 strain JEV (ip).

double = two inoculations with 10^7 pfu vaccinia recombinants (ip) 3 weeks apart and challenge 3 weeks later with 1.3×10^5 LD₅₀ P3 strain JEV (ip).

Table 5. Post challenge immune response

Inoculations	vP555	vP829	vP825	vP857	vP864
single	++	+	++	- ^a	++++
double	+/- ^b	-	-	++	+++

+ NS3 antibodies present in post-challenge sera

a No surviving mice

b Very low level NS3 antibodies present in post-challenge sera

Example 10 - CLONING OF JEV GENES INTO A VACCINIA (NYVAC) DONOR PLASMID

Plasmid pMP2VCL (containing a polylinker region within vaccinia sequences upstream of the K1L host range gene) was digested within the polylinker with HindIII and XhoI and ligated to annealed oligonucleotides SPHPRHA A through D generating

SPHPRHA A (SEQ ID NO:31) 5'-

AGCTTCTTTATTCTATACTAAAAAGTGAAAATAACAAAGGTTCTTGAGGGT - 3'

SPHPRHA B (SEQ ID NO:32) 5'-

TGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTCATTATCGCGATATCCGTTAA
GTTTGTATCGTAC - 3'

SPHPRHA C (SEQ ID NO:33) 3'-

TTATTAGTATTTAATAAAAGTAATAGCGCTATAGGCAATTCAAACATAGCATGA
GCT - 5'

SPHPRHA D (SEQ ID NO:34) 3' -

AGAAATAAGATATGAATTTTCACTTTATTATGTTCCAAGAACTCCAACACAATT
AACTTTCGCTCT - 5'

SP126 containing a HindIII site, H6 promoter -124 through -1 (Perkus et al., 1989) and XhoI, KpnI, SmaI, SacI and EcoRI sites.

Plasmid pSD544VC (containing vaccinia sequences surrounding the site of the HA gene replaced with a polylinker region and translation termination codons in six reading frames) was digested with XhoI within the polylinker, filled in with the Klenow fragment of DNA polymerase I and treated with alkaline phosphatase. SP126 was digested with HindIII, treated with Klenow and the H6 promoter isolated by digestion with SmaI. Ligation of the H6 promoter fragment to pSD544VC generated SPHA-H6 which contained the H6 promoter in the polylinker region (in the direction of HA transcription).

Plasmid JEV14VC (FIG. 1) was digested with EcoRV in the H6 promoter and SacI in JEV sequences (nucleotide 2124) and a 1789 bp fragment isolated. JEV14VC was digested with EclXI at the EagI site following the T5NT, filled in with the Klenow fragment of DNA polymerase I and digested with SacI in JEV sequences (nucleotide 2124)

generating a 2005 bp fragment. The 1789 bp EcoRV-SacI and 2005 bp (SacI-filled EclXI) fragments were ligated to EcoRV (within H6) and SmaI digested (within polylinker) and alkaline phosphatase treated SP126 generating JEV35. JEV35 was transfected into vP866 (NYVAC) infected cells to generate the vaccinia recombinant vP908 (FIG. 18).

JEV35 was digested with SacI (within JE sequences nucleotide 2124) and EclXI (after T5NT) a 5497 bp fragment isolated and ligated to a SacI (JEV nucleotide 2125) to EagI fragment of JEV25 (containing the remaining two thirds of E, translation stop and T5NT) generating JEV36. JEV36 was transfected into vP866 (NYVAC) infected cells to generate the vaccinia recombinant vP923 (FIG. 18).

Oligonucleotides SPHPRHA A through D (SEQ ID NO:31), (SEQ ID NO:32), (SEQ ID NO:33) and (SEQ ID NO:34) are ligated to generate the following sequences (SEQ ID NO:56/SEQ ID NO:57)

HindIII
A+B 5'- AGCTTCTTATTCTATACTTAAAAAGTGAAAATAACAAAGGTTCTTGAG
D+C 3'- AGAAATAAGATATGAATTTTCACTTTTATTATGTTCCAAGAAC
20 GGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTCATTATCGC
CCAACACAATTAACTTCTCGCTTTATTAGTATTAAATAAGTAATAGCG

EcoRV
25 GATATCCGTTAAGTTGTATCGTAC -3' A+B
CTATAGGCAATTCAAACATAGCATGAGCT -5' D+C
XbaI

Animal Protection Experiment

Mouse protection experiments were performed exactly as described by Mason et al. (1991). Groups of 3 week old mice were immunized by intraperitoneal (ip) injection of 10⁷ pfu of vaccinia virus, and 3 weeks later sera were collected from selected mice. Mice were then challenged by ip injection with a suspension of suckling mouse brain infected with the P3 strain of JEV (multiple mouse passage; Huang, 1982). Following challenge mice were observed daily for three weeks.

Evaluation of Immune Response to JEV NYVAC Recombinants

Hemagglutinin inhibition (HAI) tests were performed as described by Mason et al. (1991).

Vaccination with JEV NYVAC Recombinants Provided Protection from Lethal JEV Infection

NYVAC recombinants vP908 and VP923 elicited high levels of hemagglutination-inhibiting antibodies and protected mice against more than 100,000 LD₅₀ of JEV (Table 6).

10 Table 6. Ability of JEV NYVAC recombinants to protect mice from lethal JEV encephalitis

	Immunizing Virus	Pre-challenge	Survival/total
	NYVAC (vP866)	<1:10	0/12
20	vP908	1:80	11/12
	vP923	1:80	10/10

25 Immunization - one inoculation of 10⁷ pfu, ip route.

Challenge - 3 weeks post immunization 3.8 × 10⁵ LD₅₀ P3 strain JEV ip route

30 Example 11 - CLONING OF YF GENES INTO A VACCINIA VIRUS DONOR PLASMID

A host range mutant of vaccinia virus (WR strain) vP293 (Perkus et al., 1989), was used to generate all recombinants (see below). All vaccinia virus stocks were produced in either VERO (ATCC CCL81) or MRC-5 (ATCC CCL171) cells in Eagles MEM supplemented with 5-10% newborn calf serum (Flow Laboratories, McLean, VA).

40 The YF 17D cDNA clones used to construct the YF vaccinia recombinant viruses (clone 10III and clone 28III), were obtained from Charles Rice (Washington University School of Medicine, St. Louis, MO), all nucleotide coordinates are derived from the sequence data presented in Rice et al., 1985.

45 Plasmid YF0 containing YF cDNA encoding the carboxy-terminal 80% prM, E and amino-terminal 80% NS1

(nucleotides 537-3266) was derived by cloning an AvaI to NsiI fragment of YF cDNA (nucleotides 537-1658) and an NsiI to KpnI fragment of YF cDNA (nucleotides 1659-3266) into AvaI and KpnI digested IBI25 (International Biotechnologies, 5 Inc., New Haven, CT). Plasmid YF1 containing YF cDNA encoding C and amino-terminal 20% prM (nucleotides 119-536) was derived by cloning a RsaI to AvaI fragment of YF cDNA (nucleotides 166-536) and annealed oligos SP46 and SP47 (containing a disabled HindIII sticky end, XhoI and ClaI 10 sites and YF nucleotides 119-165) into AvaI and HindIII digested IBI25. Plasmid YF3 containing YF cDNA encoding the carboxy-terminal 60% of E and amino-terminal 25% of NS1 was generated by cloning an ApaI to BamHI fragment of YF cDNA (nucleotides 1604-2725) into ApaI and BamHI digested IBI25. 15 Plasmid YF8 containing YF cDNA encoding the carboxy-terminal 20% NS1 NS2A, NS2B and amino-terminal 20% NS3 was derived by cloning a KpnI to XbaI fragment of YF cDNA (nucleotides 3267-4940) into KpnI and XbaI digested IBI25. Plasmid YF9 containing YF cDNA encoding the carboxy-terminal 60% NS2B 20 and amino-terminal 20% NS3 was generated by cloning a SacI to XbaI fragment of YF cDNA (nucleotides 4339-4940) into SacI and XbaI digested IBI25. Plasmid YF13 containing YF cDNA encoding the carboxy-terminal 25% of C, prM and amino-terminal 40% of E was derived by cloning a BalI to ApaI 25 fragment of YF cDNA (nucleotides 384-1603) into ApaI and SmaI digested IBI25.

Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to change potential vaccinia virus early transcription termination signals (Yuen et al., 1987) 49 aa 30 from the amino-terminus of the C gene in YF1 (TTTTTCT nucleotides 263-269 and TTTTTGT nucleotides 269-275) to (SEQ ID NO:35) TTCTTCCTTCTTGT creating plasmid YF1B, in the E gene 35 in YF3 (nucleotides 1886-1893 TTTTTTGT to TTCTTTGT 189 aa from the carboxy-terminus and nucleotides 2429-2435 TTTTTGT to TTCTTG 8 aa from the carboxy-terminus) creating plasmids YF3B and YF3C. A PstI to BamHI fragment from YF3C (nucleotides 1965-2725) was exchanged for the corresponding

fragment of YF3B generating YF4 containing YF cDNA encoding the carboxy-terminal 60% E and amino-terminal 25% NS1 (nucleotides 1604-2725) with both mutagenized transcription termination signals. An ApaI to BamHI fragment from YF4 (nucleotides 1604-2725) was substituted for the equivalent region in YF0 creating plasmid YF6 containing YF cDNA encoding the carboxy-terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides 537-3266) with both mutagenized transcription termination signals. Plasmid YF6 was digested 10 with EcoRV within the IBI25 sequences and AvaI at nucleotide 537 and ligated to an EcoRV to AvaI fragment from YF1B (EcoRV within IBI25 to AvaI at nucleotide 536) generating YF2 containing YF cDNA encoding C through the amino-terminal 80% of NS1 (nucleotides 119-3266) with an XhoI and ClaI site 15 at 119 and four mutagenized transcription termination signals.

Oligonucleotide-directed mutagenesis described above was used to insert XhoI and ClaI sites preceding the ATG 17 aa from the carboxy-terminus of E (nucleotides 2402-2404) in plasmid YF3C creating YF5, to insert XhoI and ClaI sites preceding the ATG 19 aa from the carboxy-terminus of prM (nucleotides 917-919) in plasmid YF13 creating YF14, to insert an XhoI site preceding the ATG 23 aa from the carboxy-terminus of E (nucleotides 2384-2386) in plasmid 25 YF3C creating plasmid YF25, and to insert an XhoI site and ATG (nucleotide 419) in plasmid YF1 21 aa from the carboxy-terminus of C generating YF45.

An ApaI to BamHI fragment from YF5 (nucleotides 1604-2725) was exchanged for the corresponding region of YF0 creating YF7 containing YF cDNA encoding the carboxy-terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides 537-3266) with XhoI and ClaI sites at 2402 (17 aa from the carboxy-terminus of E) and a mutagenized transcription termination signal at 2429-2435 (8 aa from the carboxy-terminus of E). The ApaI to BamHI fragment from YF25 (nucleotides 1604-2725) was exchanged for the corresponding region of YF0 generating YF26 containing YF cDNA encoding

the carboxy-terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides 537-3266) with an XhoI site at nucleotide 2384 (23 aa from the carboxy-terminus of E) and mutagenized transcription termination signal at 2428-2435 (8 aa from the 5 carboxy-terminus of E).

An AvaI to Apal fragment from YF14 (nucleotides 537-1603) was substituted for the corresponding region in YF6 generating YF15 containing YF cDNA encoding the carboxy-terminal 80% prM, E and amino-terminal 80% NS1 (nucleotides 10 537-3266) with XhoI and ClaI sites at nucleotide 917 (19 aa from the carboxy-terminus of prM) and two mutagenized transcription termination signals. YF6 was digested within IBI25 with EcoRV and within YF at nucleotide 537 with AvaI and ligated to EcoRV (within IBI25) to AvaI fragment of YF45 15 generating YF46 containing YF cDNA encoding C through the amino-terminal 80% NS1 (nucleotides 119-3266) with an XhoI site at 419 (21 aa from the carboxy-terminus of C) and two transcription termination signals removed.

Oligonucleotide-directed mutagenesis described 20 above was used to insert a SmaI site at the carboxy-terminus of NS2B (nucleotide 4569) in plasmid YF9 creating YF11, and to insert a SmaI site at the carboxy-terminus of NS2A (nucleotide 4180) in plasmid YF8 creating YF10. A SacI to 25 XbaI fragment from YF11 (nucleotides 4339-4940) and Asp718 to SacI fragment from YF8 (nucleotides 3262-4338) were ligated to Asp718 and XbaI digested IBI25 creating YF12, containing YF cDNA encoding the carboxy-terminal 20% NS1, NS2A, NS2B and amino-terminal 20% NS3 (nucleotides 3262-4940) with a SmaI site after the carboxy-terminus of NS2B 30 (nucleotide 4569).

Plasmid pHES4 contains the vaccinia K1L host range gene, the early/late vaccinia virus H6 promoter, unique multicloning restriction sites, translation stop codons and an early transcription termination signal (Perkus et al., 35 1989). A KpnI to SmaI fragment from YF12 encoding carboxy-terminal 20% NS1, NS2A and NS2B (nucleotides 3267-4569), XhoI to KpnI fragment from YF15 encoding 19 aa prM, E and

amino-terminal 80% NS1 (nucleotides 917-3266) and XhoI-SmaI digested pHES4 were ligated generating YF23. An XhoI to BamHI fragment from YF26 encoding 23 aa E, amino-terminal 25% NS1 (nucleotides 2384-2725) was ligated to an XhoI to 5 BamHI fragment from YF23 (containing the carboxy-terminal 75% NS1, NS2A and NS2B, the origin of replication and vaccinia sequences) generating YF28.

XhoI-SmaI digested pHES4 was ligated to a purified XhoI to KpnI fragment from YF7 encoding 17 aa E and amino-terminal 80% NS1 (nucleotides 2402-3266) plus a KpnI to SmaI fragment from YF10 encoding the carboxy-terminal 20% NS1 and NS2A (nucleotides 3267-4180) creating YF18. An XhoI to BamHI fragment from YF2 encoding C, prM, E and amino-terminal 25% NS1 (nucleotides 119-2725) was ligated to a 15 XhoI to BamHI fragment of YF18 (containing the carboxy-terminal 75% NS1 and NS2A, the origin of replication and vaccinia sequences) generating YF19. The same XhoI to BamHI fragment from YF2 was ligated to a XhoI to BamHI fragment from YF28 (containing the carboxy-terminal 75% NS1 and NS2A, the origin of replication and vaccinia sequences) generating 20 YF20. A XhoI to BamHI fragment from YF46 encoding 21 aa C, prM, E and amino-terminal 25% NS1 (nucleotides 419-2725) was ligated to the XhoI to BamHI fragment from YF18 generating 25 YF47. Oligonucleotide SP46 (SEQ ID NO:36) and SP47 (SEQ ID NO:37) are as follows:

HindIII

SP46 5' - AGCTT CTCGAGCATCGATTACT atg TCTGGTCGTAAAGCTCAGGGA
SP47 3' - A GAGCTCGTAGCTAATGA TAC AGACCAGCATTTCGAGTCCCT

30 AAAACCTGGCGTCAATATGGT -3'
TTTGGGACCCGCAGTTATACCA -5'

Construction of Vaccinia Recombinants

Procedures for transfection of recombinant donor plasmids into tissue culture cells infected with a rescuing 35 vaccinia virus and identification of recombinants by host range selection and *in situ* hybridization on nitrocellulose filters have been described (Perkus et al., 1989). YF18, YF23, YF20, YF19 and YF47 were transfected into host range mutant vP293 (Perkus et al. 1989) infected cells to generate

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